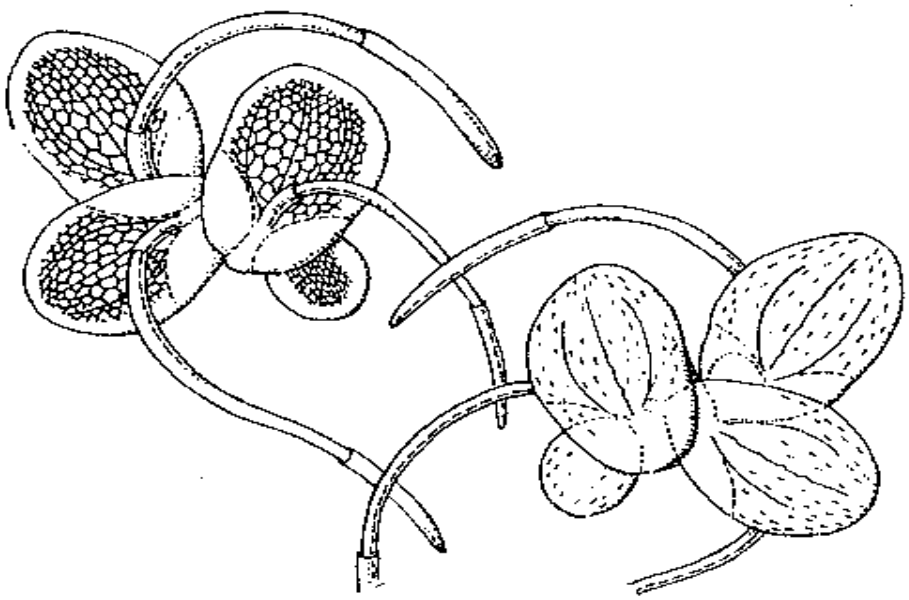
THE FACTOR TIME IN ASSESSING ECOTOXICITY

Studies with Herbicides and Metals applied singly and in combination to *Lemna minor* in simple and complex exposure patterns.



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Studies with Herbicides and Metals applied singly and in combination to *Lemna minor* in simple and complex time patterns.

DER FAKTOR ZEIT BEI DER ABSCHÄTZUNG DER ÖKOTOXIZITÄT

Untersuchungen mit Herbiziden und Metallen einzeln und in Kombination appliziert auf *Lemna minor* in einfachen und komplexen Zeitmustern

Dissertation zur Erlangung des naturwissenschaftlichen Doktorgrades Dr. rer. nat.

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List of abbreviations

Acute to Chronic Ratios	ACR
Atomic Absorption Spectrometer	AAS
Concentration Addition	CA
Critical Area Under the Curve	CAUC
Critical Body Residue	CBR
Critical Target Occupation Model	CTO
Damage Assessment Model	DAM
Dynamic Energy Budget concept	DEB
Dynamic Energy Budget Toxicity model	DEBtox
effect concentration eliciting 1% effect	EC_1
effect concentration eliciting 50% effect	EC_{50}
Flow Injection Analysis	FIA
High Liquid Chromatography	HPLC
Independent Action	IA
Inductively-Coupled-Plasma Optical-Emission-Spectrometer	ICP-OES
International Organization for Standardization	ISO
Lowest Observed Effect Concentration	LOEC
Mechanism of Action	MeoA
Mode of Action	MoA
Multi-Component Damage Assessment Model	MDAM

No Effect Concentration	NEC
No Observed Effect Concentration	NOEC
Organisation for Economic Co-operation and Development	OECD
Polycyclic Aromatic Hydrocarbons	PAHs
Predicted Environmental Concentration	PEC
Predicted No Observed Effect Concentration	PNEC
protoporphyrinogen IX	PPGIX
Regulation, Evaluation, Authorisation and Restriction of Chemicals	REACH
Reactive Oxygen Species	ROS
Threshold Damage Model	TDM
Toxicity Exposure Ratios	TER
Water Frame Directive	WFD

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Summary

Ecotoxicological toxicity data serve as a basis to assess the hazard of a substance and help to decide whether a risk of adverse effects in the environment is likely. Toxicity tests are often restricted to simple single substance tests with short time periods of exposure. They only regard toxicity at a certain time point distinguishing only between acute or chronic toxicity. However, time consists of many timescales, like toxicodynamic, the dynamics of injury and recovery and toxicokinetic, the dynamics of absorption and elimination of a toxicant. Additionally, the exposure to hazardous compounds may not follow simple exposure patterns. Environmental concentrations of pollutants may fluctuate, due to variable emissions from anthropogenic sources such as the seasonal use of herbicides and storm runoff or flooding. As a result, organisms can be exposed to a multitude of different toxicants with complex time patterns.

Taking these aspects into consideration, the aim of this study is to gain an insight into what influence time has on toxicity and how toxicity develops if the exposure follows complex time patterns. As a test organism *Lemna minor*, a limnic vascular plant was used due to its ecological importance and its convenience for the test pattern of this presented research. Test substances with different Mode of Actions were the herbicides Alachlor, Aclonifen, Ametryn, Prometon, Diuron and Paraquat. The tested metals were zinc, copper, cadmium and nickel. All these substances are of importance as environmental pollutants.

Single substance toxicity over a set length of time was investigated for each substance. Concentration-response relationships were recorded at different points of time over a total of seven days. The results show that toxicokinetics as well as toxicodynamics are important and they are concentration-dependent and specific for each substance and its Mode of Action. This work stresses that it is important to consider toxicodynamic properties, which are often neglected in studies on toxicity and time.

In the case of the metals, their uptake over time into the plants was investigated via AAS. It was shown that concerning the toxicity of the metals it is crucial how well a toxicant is absorbed. The internal concentrations of the metals were quite similar, despite their largely differing external EC_{50} values. There was however no correlation between toxicity over time and the total internal concentration over time. Instead the changing toxicity of the metals may apart from a cumulative damage be attributed to a dynamic distribution within the plant.

Though simple, the empiric Haber's law and its derivations gave a good description of the toxicity-time relationship of the obtained data and thus may be a good tool to extrapolate toxicity for different time points in environmental hazard assessment. Additionally, the data fitted equations give information on whether the toxicity of a substance is more concentration-or time-dependent. The mechanistic model DEBtox, based on the assumption that toxicity is mainly determined by toxicokinetics, may be too simplistic. The results of this work show toxicokinetics and toxicodynamics are both important and should both be considered.

All substances were investigated concerning the recovery potential of the plants after a three-day pulse. Substances with a slow reversible Mode of Action/Mechanism of Action and time-dependent toxicity showed a slow concentration-dependent recovery potential, whereas substances with a quick reversible Mode of Action/Mechanism of Action showed a concentration-independent fast recovery potential.

The study with *Lemna minor* and fluctuating concentrations of Alachlor, copper or Diuron showed that the damage is either cumulative (Alachlor), that the plants are able to adapt (copper) or that the plants only show an increase of sensitivity over a short time due to fast recovery (Diuron).

The order of application was crucial for the overall effect if Alachlor, copper and Diuron were combined sequentially. Different results were observed for the same combination of substances but in a different order.

The predictive concepts Concentration Addition (CA) and Independent Action (IA) gave a good estimation of the toxicity of the mixtures studied with a constant composition. Concerning the sequential combination of Alachlor, Diuron and copper the two concepts were applicable within limits, as the sequence is not considered by these concepts.

This work shows that the time factor is important. It is not only 'the dose that makes a poison' but also the time that makes a poison. Therefore, toxicity-time relationships and more complex exposure patterns should also be considered as worthy of investigation. Studies which take these aspects into account may help to refine the risk assessment for instance of crop protection and to indentify pulse sequences that may be less harmful to the environment than other usage patterns.

Zusammenfassung

Ökotoxikologische Toxizitätstests bilden die Basis für die Gefährdungsabschätzung eines Stoffes und der Abschätzung des Risikos einer Umweltgefährdung. Toxizitätstests sind meist beschränkt auf einfache Einzelsubstanz-Tests mit kurzer Testzeit, die einen bestimmten Zeitpunkt betrachten und nur zwischen akuter und chronischer Toxizität unterscheiden. Die Zeit besteht jedoch aus unterschiedlichen Zeitverläufen wie die Toxikodynamik, die den zeitlichen Verlauf des verursachten Schadens durch eine toxische Substanz und dessen Reparatur betrachtet, und die Toxikokinetik, die den zeitlichen Verlauf der Aufnahme einer Substanz und Ausscheidung betrachtet. Zusätzlich kommen Chemikalien nicht einzeln vor und die tatsächlichen Umweltkonzentrationen von Chemikalien schwanken. Dies wird z.B. verursacht durch die saisonale Verwendung von Pflanzenschutzmitteln, punktuelle kurze Emission von Chemikalien, Strömungen, oder Regenfälle, die Schadstoffe in Gewässer eintragen. Das heißt, Organismen sind einer Vielzahl verschiedener Chemikalien mit komplexen Zeitmustern ausgesetzt.

Unter Berücksichtigung der oben genannten Aspekte ist es das Ziel dieser Arbeit Einblick zu gewinnen, welchen Einfluss der Faktor Zeit auf die Toxizität und wie sich die Toxizität entwickelt wenn die Exposition gegenüber Stoffen komplexeren Zeitmustern folgt. Als Testorganismus wurde die limnische vaskuläre Pflanze *Lemna minor* verwendet. Diese Pflanze ist einerseits ökologisch wichtig aber auch für das Versuchsdesign bei dieser Arbeit besonders gut geeignet. Als Testsubstanzen mit unterschiedlichen Wirkweisen wurden die Metalle Zink, Kupfer, Cadmium und Nickel sowie die Pflanzenschutzmittel Alachlor, Aclonifen, Ametryn, Prometon, Diuron und Paraquat untersucht.

Der zeitliche Verlauf der Einzelstofftoxizität wurde für jede Substanz untersucht, indem die Konzentrations-Wirkbeziehung zu mehreren Zeitpunkten über sieben Tage bestimmt wurde. Diese Arbeit zeigt dass, die Toxikokinetik und die Toxikodynamik beide wichtig, konzentrationsabhängig und für jede Substanz und seine Wirkweise spezifisch sind. Die Ergebnisse dieser Arbeit betonen, dass es wichtig ist auch die Toxikodynamik in Betracht zu beziehen. Dies wird oft vernachlässigt bei der Toxizität über die Zeit.

Bei der Untersuchung der Metalle wurde zusätzlich mittels AAS der Verlauf der Metallaufnahme über die Zeit gemessen. Hier zeigte sich, dass die Toxizität der Metalle vor Allem durch ihre Aufnahme bestimmt wird. Die internen Konzentrationen der Metalle in den Pflanzen waren ähnlich, während die ermittelten externen EC_{50} Werte sehr unterschiedlich waren. Andererseits ließ sich die verändernde Toxizität über die Zeit nicht durch eine

veränderte interne Gesamtkonzentration erklären, sondern ist eher einer dynamische Verteilung der Metallkonzentration über die Zeit innerhalb der Pflanze und kumulativen Schäden zuzuschreiben.

Es gibt unterschiedliche Ansätze, entweder empirische oder mechanistische, um die Toxizitäts-Zeit-Beziehung zu beschreiben. Obwohl ein sehr einfacher Ansatz, erwies sich das empirische Habersche Gesetz sowie dessen Variationen als eine gute Beschreibung der Toxizitäts-Zeit-Beziehung und könnte daher ein gutes Mittel sein, um bei der Gefährdungsabschätzung eines Stoffes die Toxizität über die Zeit zu extrapolieren. Das mechanistische Modell DEBTox basiert auf der Annahme, dass die Toxizität hauptsächlich durch die Toxikokinetik bestimmt wird. Aufgrund der in dieser Arbeit erstellten Daten muss jedoch geschlossen werden, dass diese Annahme zu einfach ist. Sowohl die Toxikokinetik als auch die Toxikodynamik sind beide wichtig und sollten daher beide in Betracht gezogen werden.

Alle Substanzen wurden hinsichtlich ihres Erholungspotentials nach einer dreitägigen Exposition untersucht. Substanzen mit einem langsam reversiblen Wirkmechanismus und einer zeitbestimmten Toxizität zeigten eine langsame konzentrationsabhängige Erholung, während Substanzen mit einem schnell reversiblen Wirkmechanismus ein konzentrationsunabhängiges schnelles Erholungspotential zeigten.

Untersuchungen mit *Lemna minor* und fluktuierenden Konzentrationen von Alachlor, Kupfer oder Diuron zeigten, dass der verursachte Schaden entweder kumulativ (Alachlor) ist, die Pflanzen sich anpassen (Kupfer) oder nur eine kurze Phase erhöhter Empfindlichkeit aufgrund des schnellen Erholungspotentials beobachtbar ist (Diuron).

Die Sequenz ist wichtig, wenn Substanzen sequentiell miteinander kombiniert werden. Diese zeigen die Untersuchungen dieser Arbeit wenn Alachlor, Kupfer und Diuron miteinander kombiniert wurden. Unterschiedliche Ergebnisse konnten beobachtet werden bei der gleichen Kombination aber einer unterschiedlichen Sequenz.

Die Vorhersagekonzepte Konzentrationsadditivität (CA) und Unabhängige Wirkung (IA) lieferten gute Vorhersagen der Mischungstoxizität von untersuchten Mischungen mit einer konstanten Zusammensetzung. Wurden Alachlor, Kupfer und Diuron jedoch sequentiell miteinander kombiniert waren diese Vorhersagekonzepte nur bedingt anwendbar, da die Konzepte die Sequenz nicht berücksichtigen.

Diese Arbeit zeigt: Zeit ist wichtig. Nicht nur ‚die Dosis macht das Gift' sondern auch die Zeit macht das Gift. Daher sollten Toxizitäts-Zeit-Beziehung und komplexere Expositionsmuster bei Untersuchungen ebenfalls in Betracht gezogen werden. Untersuchungen, die diese Aspekte berücksichtigen, könnten zum Beispiel zur Verfeinerung der Risikobewertung von Pflanzenschutzmitteln und ihrer Anwendung beitragen und solche Pulssequenzen identifizieren, die weniger schädigend auf die Umwelt wirken.

1 Introduction

1.1 Environmental pollution and the assessment of its hazards

Due to their beneficial properties chemicals have found a broad acceptance and are being used in our everyday life in homes and workplaces as well as in agricultural and industrial production. The profitable use of metals as alloys or salts dates back to the Iron Age. In comparison, the beneficial use of synthetic organic compounds started just before and especially after World War II when synthetic chemistry rapidly evolved and provided almost boundless possibilities for developing new chemicals (Jastorff et al., 2003). However, apart from their advantages, chemicals also pose a variety of problems. This is not an exclusively modern concern. Though not on a modern scale, even in Greco-Roman times for instance pollution was caused due to unreduced effluents. Apart from wastes, smoke and odour, pollutants of that time that were released from gold refineries or separated from silver ore were lead and mercury. As ingredients of paints and dyes, arsenic and lead were part of daily used products. Hughes (1994) assumes that such pollution may even have been a contribution to the decline of these cultures.

The awareness of the negative impacts of synthetic chemicals lagged behind the evolution of synthetic chemistry and increased in the sixties of the last century. This awareness was substantiated by incidences such as accidental spills as in the case of Seveso (1976). Apart from risks of chemicals on humans, Rachel Carson's book 'Silent spring' published in 1962 deals with the impact of the organochlorine pesticides DDE, DDT and DDD on humans and wildlife, which triggered a growing interest in the emerging field of environmental toxicology (Walker, 2006) and is often believed to be the naissance of environmental awareness (Winiwarter and Knoll, 2007). Up until the sixties there was only a minimal awareness of ecological problems related to the release of chemicals into the environment and little attention had been paid to environmental risk assessment whereas the risk assessment of toxicants on humans was relatively well researched (Walker, 2006).

1.1.1 Current status of the regulation of chemicals

Nowadays, several legislative acts have been set into force in order to manage the uses of chemicals and to restrict their danger to humans and the environment. For instance, the new European regulation for the "Regulation, Evaluation, Authorisation and Restriction of Chemicals"(REACH) inaugurated in June 2007 considers industrial chemicals and created a single system for industrial chemicals which had been regulated unequally depending on the date they had been introduced onto the market (Council of the European Communities, 2006).

The directive 91/414/EEC regulates plant protection products such as herbicides (Council of the European Communities, 1991). To regain and retain a good environmental status of waterbodies the Water Frame Directive (WFD) has been inaugurated (European Parliament et al., 2001).

Environmental protection and management strategies rely on the estimation of the hazard a chemical may pose on ecosystems (Forbes and Forbes, 1994). Ideally, tests designed to assess the hazard of a chemical should reflect the situation in the environment as realistically as possible. However, this conflicts with practical constraints such as limited resources (budget, time, manpower) and social issues (e.g. animal welfare) (Junghans et al., 2008). Also the sheer number of chemicals which need to be assessed and the complexity of the environment make an in-depth risk assessment unfeasible. In addition to that, the regulation of chemicals favours clear and decisive results of standardised tests. Hence, effect assessment is generally restricted to simple standardized toxicity experiments although environmental risk assessment aims to protect the structure and functioning of ecological systems of higher biological organisation (Junghans et al., 2008). A test system consists of one species as a representative of an organism, of a compartment such as freshwater and representative of a trophic level. Based on the toxicity data of these representative organisms which are mainly fish, algae and daphnids, a predicted no observed effect concentration (PNEC) is generated. Depending on the uncertainty an additional uncertainty or assessment factor may be applied. The PNEC-value is compared with the predicted environmental concentration (PEC), which is based on an exposure assessment of the substance regarded. Based on the obtained PEC/PNEC ratios, a quantitative risk-characterization is carried out. The risk assessment of pesticides is performed in a particular way. In contrast to the estimation of PEC/PNEC ratios, so-called “toxicity exposure ratios” (TER) are estimated (EC_{50}/PEC) to determine whether the risk to an organism is acceptable or not.

In regulation substances are generally regarded singly and the tests conducted to assess the toxicity of a substance refer to a certain time point either determining acute or chronic toxicity. How toxicity evolves over time under simple or complex exposure scenarios is generally not the subject of the hazard assessment of chemicals. This approach however hardly describes the full consequence of a toxic substance on something as complex as an ecosystem nor does it sufficiently reflect the real site situation of hazardous chemicals in the environment either released on purpose or accidentally. Referring to the use and release of chemicals into the environment, organisms are exposed to a cocktail of pollutants in

concentrations with long time-scales or complex time patterns. However, to assess the hazard of each pollutant considering the different time scales and time patterns of its occurrence in the environment is not feasible. Nor is it practicable to assess the mixture toxicity of each possibly occurring mixture. In order to circumvent the conduction of additional testing due to practical constraints and to tackle this problem, approaches try to extrapolate, model or predict the hazard of such real site situations. Those approaches, which are the subject of this work, will be introduced in the following chapters.

1.2 The relation between toxicity and time

Time can be regarded as the time point or time course. Both play an important role in toxicity. Chronotoxicity and chronopharmacology deal with the time point a toxicant is applied. This field of toxicology deals with time-of-day effects and takes into account the chronobiology of organisms. The term chronopharmacology was coined in the early 1960s and especially investigates the efficiency pharmaco-therapeutics or the toxicity of chemicals in workplaces depending on the time of day (Dhami et al., 1997).

Focusing on the time course, toxicity depends on exposure, which is a function of dose and time. Though both parameters have the same importance, toxicologists have rather focused on the dependency of dosage and toxicity than time and toxicity (Rozman and Doull, 2000).

Time is often only regarded in a semi quantitative manner, distinguishing between acute and chronic toxicity. Compared to dosage, which is a simple variable as it only depends on the number of molecules, the variable time is multidimensional. Time consists of many timescales, like toxicodynamics, toxicokinetics and frequency and duration of exposure (Rozman, 2000). Toxicokinetics deals with the dynamic change between absorption and elimination of a toxicant, whereas toxicodynamics studies the dynamic change between injury and recovery. Absorption occurs faster than elimination and injury occurs faster than repair. Thus, elimination, which includes the processes of excretion, distribution and biotransformation, determines toxicokinetics. In analogy, recovery, which includes reversibility, repair and determines the toxicodynamics (Rozman and Doull, 2000).

There are several different approaches, which try to describe toxicity over time. Time to event models approach time and toxicity by determining a time point including duration and intensity of exposure at which a defined response occurs (Crane et al. 2002; Karman 2000 et al.).Other models assume a time-dependent or time-independent threshold. The idea is that a toxicant causes an effect if the internal concentration in an organism of the toxicant exceeds

this threshold. (Kooijman, 2000b; Legierse et al., 1999; Verhaar et al., 1999; Bedaux and Kooijman, 1994). Another approach additionally includes the damage a toxicant causes in order to describe the toxicity over time (Lee J-H et al., 2002). Apart from these refined mechanistic models, there are also approaches, which describe the toxicity-time empirically (Ahlers et al., 2006; Länge et al., 2004; Rozman and Doull, 2000).

In this work, special focus is put onto the approach of Rozman and Doull, who promote the relatively simple approach of Haber and Bliss. This simple empirical approach will be compared to and discussed with the mechanistic approach of Kooijman, who developed the dynamic energy budget theory (DEB) and its spinoff the Dynamic Energy Budget Toxicity model (DEBtox).

1.2.1 Haber's Rule and its derivations

Rozman and Doull are advocates for an extrapolation method which dates back to 1900 (Rozman and Doull, 2000; Schramm et al., 2002; Rozman, 2005; Rozman and Doull, 2001a; Rozman and Doull, 2001b; Rozman 2000). Warren quantitatively linked the relationship between dose and time the first time (Rozman and Doull, 2001b; Warren, 1900). Ostwald and Dernoscheck (1910) modified Warren's formula in analogy with the adsorption isotherm to

$$c^{\gamma} \bullet t = k. \qquad (1)$$

In this equation c is the concentration, which is exponentiated by the power term γ and multiplied by the exposure-time t. The product is constant which is expressed by the constant k.

Haber (1924) used the simplest version of dose/time relationship.

$$c \bullet t = k \qquad (2)$$

Though this equation was only mentioned in a footnote by Haber, the equation became 'Haber's rule' and was most frequently confirmed by entomologists (Rozman, 2000).

Bliss (1940) concluded from his observations that departures from the Haber equation can be either described by the equation introduced by Ostwald and Dernoscheck (1910) or by introducing a power term for the time (t).

$$c \bullet t^{\gamma} = k \qquad (3)$$

If the hyperbolic equations are logarithmically transformed or plotted on log-log paper, the equations become linear and thus can be easily solved by hand.

Though these equations are simple, they demand some experimental preconditions as Rozman illustrates (Rozman 2000). According to Rozman, these preconditions should be fulfilled for aquatic ecotoxicological experiments and therefore Haber's rule or the derivations from it should be suitable to describe the change of toxicity over time. Whether this is the case, will be investigated. Additionally, these equations may give insight into which extent toxicity is time- or concentration-dependent as stated by Miller et al. (Miller et al., 2000), which may be linked to the Mode of Action (MoA) of the tested substance.

1.2.2 The DEBtox approach

Compared to the approach for which Rozman and Doull strongly stand up for and which can be easily solved by hand on log-log paper, the Dynamic Energy Budget Toxicity model (DEBtox) is mathematically more challenging and thus needs considerable processor capacity (Bedaux and Kooijman, 1994; Kooijman, 2000b). The aim of DEBtox is the derivation of a threshold value which is called the No Effect Concentration (NEC). This NEC is a model parameter and is the concentration of the chemical which does not cause any effect after prolonged exposure. The NEC has been suggested as an alternative to the time-dependent No Observed Effect Concentration (NOEC). In contrast, the NOEC is not a model parameter but one of the test concentrations of the regarded test and always linked to the test duration. This value is important concerning the regulation of chemicals as it used for the derivation of the PNEC.

DEBtox is based on the Dynamic Energy Budget concept (DEB), which deals with the acquisition of energy by an individual organism and its utilisation for growth, reproduction and survival. Finding similarities between different organisms, Kooijman, the founder of this theory, tried to establish a mathematical framework which captures the energy budget of such organisms as diverse as 'zebras, beetles and bacteria'. The organisms are treated as a dynamic system with a mass and energy balance. This framework is described in detail in (Kooijman, 2000a). The DEBtox model was developed as a by-product of the DEB theory. According to DEBtox a toxicant disturbs and changes the energy balance of an organism. The NEC concept is based on the idea that each molecule may have an effect, thus the NEC is zero on the molecular level. However according to the model, on the level of the organism regulation systems cancel these effects out to some extent and thus the NEC is not zero. Hence, if the external concentration is smaller than the NEC, no effect is observable. According to the

model, toxicity is determined by the concentration of the toxicant alone. It is assumed that the hazard rate is proportional to the concentration of the toxicant in the organism if the internal concentration exceeds this internal threshold concentration. The DEBtox model is based on first order kinetics. The uptake rate is proportional to the environmental concentration and the elimination rate is proportional to the toxicant density in the body. A detailed mathematical description of the model has been provided by Bedaux and Kooijman (1994).

1.3 Variable exposure patterns: Pulsed and fluctuating exposure

As investigations (Kreuger, 1998; Reinert et al., 2002) of the aquatic environment have shown, the environmental concentrations of hazardous substances in the environment are not continuous but fluctuate. This is due to the seasonal use of pesticides and the resulting surface runoff, spills, spray drift, and herbicide loss from agricultural fields after rainfall. But this may not only be the case for substances released intentionally into the environment. Concentrations of heavy metals in the environment also fluctuate, due to e.g. tidal currents, storm runoff flooding or changes in speciation (Alagarsamy, 2006; Shomar et al., 2005; Montes-Botella and Tenoria, 2003; Broman et al., 1994).

Periods of intense exposure might be followed by episodes with a relatively low or no exposure. Thus the risk assessment based on continuous exposure may not provide an appropriate estimate of a toxic effect as organism may be exposed to toxicants present in pulses. Under these circumstances if a single toxicant pulse occurs, organism may recover. Many authors have thus proposed to additionally conduct recovery experiments (Reinert et al., 2002; Van Straalen et al., 1992; McCahon and Pascoe, 1990; Wright, 1976). Investigations on the recovery potential are often conducted in the context of directive 91/414/EEC concerning the placing of plant protection products on the market (Council of the European Communities, 1991). Nevertheless, even the ecotoxicological hazard assessment of substances non-intentionally entering the aquatic environment, such as heavy metals, would be improved by considering the recovery potential. Consequently, the Guidelines for Ecological Risk Assessment of the U.S: EPA, suggest not only to consider the nature and intensity of potential effects and its spatial as well as temporal scales, but also the potential for recovery (US Environmental Protection Agency, 1998).

The damage a toxic substance causes may be a crucial issue for the recovery potential. Apart from ‘How much damage?’ which is linked to the dosage, ‘What kind of damage?’ may also be important to quantify the recovery potential. Thus, this work investigates the recovery potential from exposure pulses to substances causing different physiological damages and

different exposure concentrations. Since the tests are conducted with *Lemna minor*, a vascular plant, especially herbicides, where the Mode of Action and the underlying biochemical mechanism are well investigated, are tested. Metals are included as they allow an easy investigation of the dependency of recovery on elimination or detoxification by measuring the internal metal concentration. Furthermore, this work investigates, whether repeated pulses may lead to cumulative effects or adaptation

1.4 Mixture toxicity

Apart from the exposure to single substances with complex time patterns, organisms in aquatic ecosystems are typically exposed to a multitude of pollutants, either simultaneously or sequentially. Due to the high spatial and temporal variability of the concentrations of the individual toxicants, an experimental effect assessment of all conceivable mixtures is not feasible. But predictive approaches using information on the toxicities of individual compounds may allow an estimation of the overall toxicity of a given chemical mixture. Two concepts, Concentration Addition (CA) and Independent Action (IA), can be used for such a purpose.

CA proceeds from the assumption that all components of a mixture act similarly. This concept can be traced back to the work of Frei (1913) and Loewe and Muischnek (1926) who developed this concept for two component mixtures. Berenbaum (1985) defined this concept for a mixture with n compounds as follows:

$$\sum_{i=1}^{n} \frac{c_i}{ECx_i} = 1 \qquad (4)$$

In this equation, c_i is the concentration of the *i*-th substance present in the mixture. ECx_i is the individual concentration of the *i*-th substance causing the same effect *x* as the total mixture. The quotient ci/EC_{xi} has been termed the toxic unit by Sprague (1970). The total effect of the mixture is unchanged as long as the sum of all toxic units remains constant. This implies that a mixture component can be replaced totally or in part by any other component with the same toxic unit.

The concentration of each component can be expressed as a fraction of the total concentration if the ratio of the mixture components is known. Accordingly equation 1 can be rewritten as

$$\left(\sum_{i=1}^{n} \frac{p_i}{ECx_i} \right)^{-1} = ECx_{Mix} \qquad (5)$$

EC_{mix} is the total concentration of the mixture provoking x% effect, and p_i the fraction of component i in the mixture.

In contrast, IA is based on the idea of dissimilarly acting components in a mixture with different target sites but triggering a common toxicological endpoint. This concept was first formulated by Bliss (1939) and is as the following for a two-component mixture:

$$E(c_{Mix}) = E(c_1) + E(c_2) - E(c_1) \cdot E(c_2) \qquad (6)$$

where $E(c_1)$ and $E(c_2)$ are the effects of substance 1 and 2 if applied singly in concentrations c_1 and c_2. $E(c_{mix})$ is the predicted joint effect caused by the total concentration $c_{mix} = c_1 + c_2$. For a multi-component mixture the equation can be extended as follows:

$$E(c_{Mix}) = 1 - \prod_{i=1}^{n} [1 - E(c_i)] \qquad (7)$$

$E(c_{Mix})$ is the predicted effect of a n-compound mixture, c_{Mix} is the total concentration of the mixture, c_i is the individual concentration of the *i*-th compound in the mixture and $E(c_i)$ the effect of this concentration if the compound is applied alone.

The prerequisites for the prediction of mixture toxicity are the knowledge of what substances are in the mixture as well as the single substance dose relationship of the substances. For a Concentration Addition prediction the EC values are needed whereas the Independent Action concept predicts the mixture toxicity on the basis of the effects of the single substances.

As mentioned the concept CA presumes similar acting toxicants. ‘Similar acting’ may be understood as substances with the same target site (Pöch, 1991) or substances with a common toxicological response (Berenbaum, 1985). Bliss (1939) proposed toxicants should have parallel concentration response curves if they are strictly similar but this was discarded by Plackett and Hewlett (1948). However, regardless whether the substances act similarly or dissimilarly both concepts only hold true if there are no interactions between the toxicants. Interactions may occur due to toxicokinetic or toxicodynamic interaction. Toxicokinetic interactions involve the alteration of metabolism or influence on uptake whereas toxicodynamic interactions involve a physiological alteration making the organism more or less sensitive (Plackett and Hewlett, 1948). This means the toxicity may be higher than predicted (synergistic) or lower than predicted (antagonistic).

Mixture toxicities have been successfully predicted using these concepts. Investigations have been made especially with pesticides with known and distinct Modes of Action/Mechanism of Action. It could be shown in many works with various organisms, that CA gave a good prediction of the mixture toxicity of substances with similar action (Backhaus et al., 2004; Backhaus et al., 2003; Drost et al., 2003; Junghans et al., 2003; Faust et al., 2001; Altenburger et al., 2000). For mixtures with independent acting substances IA made good predictions (Faust et al., 2003; Backhaus et al., 2000; Hermens and Leeuwangh, 1982). Though many of the investigated mixtures were designed on the basis of their EC_{50} ratios, good predictions are not restricted to this ratio, as studies with EC_1 ratios have shown (Faust e al., 2003; Altenburger et al., 2000; Grimme et al., 2000; Backhaus et al., 1997). Significant mixture effects were also observed if substances were combined in concentrations below or at their NOEC (Walter et al., 2002; Silva et al., 2002). Hence, even low effect concentrations or concentrations which are defined as not having an effect, contribute to an overall mixture toxicity. Yet, it has been stressed that the effect data have to be of good quality if predictions are based on IA (Faust et al., 2003; Altenburger et al., 2000). This is not always the case, especially for low effect concentrations. As CA overestimated the mixture toxicity of dissimilar acting substances and gave good prediction of the toxicity of similar acting components, this concept is suggested as a general solution to assess mixture toxicity on the basis of the precautionary principle (Faust et al., 2003; Faust et al., 2000).

All the above mentioned studies are artificially designed. An environmental realistic mixture based on an average European agricultural situation in springtime was investigated by Junghans et al (Junghans et al., 2006) and CA and IA gave a good assessment of the overall toxicity.

So far the concepts proved to be good prediction tools for mixture toxicity. However all studies investigated mixtures of constant qualitative and quantitative composition. This is not necessarily the case. If exposure occurs in fluctuating concentrations, organisms may be exposed to substances one at a time but nevertheless show combination effects. To which extent mixture toxicity can be assessed by the concepts IA and CA in such scenarios has not yet been analysed. It is therefore the aim of this work to analyse whether these concepts are applicable for mixtures which do not have a constant qualitative and quantitative composition.

1.5 The test organism *Lemna minor*

Lemna minor is frequently used in ecotoxicological research as a representative of higher aquatic plants. In 2006 both the OECD and ISO published final test guidelines (Organisation

for Economic Co-operation and Development (OECD), 2006; International Organization for Standardization (ISO), 2006). *Lemnas* are attractive test organisms, not only because of their important ecological functions and widespread occurrence. The plants are also easy to cultivate and handle, have a high growth rate under laboratory conditions (Lewis, 1995) and are highly sensitive to various pollutants (Fairchild et al., 1998; Fairchild et al., 1997). The plant size is small, but the fronds are large enough to be easily counted with the naked eye. This facilitates non-destructive, repeated measurements of growth patterns. The plants can be easily transferred into different media during testing which was particularly important for the studies on recovery and sequential exposure.

Lemnoideae belong to the Araceae family and comprise the *Landoltia*, *Lemna*, *Spirodela*, *Wolffia* and *Wolffiella genera*. *Lemna minor* is a limnic vascular plant commonly found in fresh water or brackish water in a cool temperate climate. Except for the southern hemisphere this plant is distributed almost worldwide (Landolt, 2009). As a part of a balanced ecosystem, they serve as an important food source for various water birds and fish and provide habitat for invertebrates (Wang, 1990). They consist of fronds floating on the surface water, which form colonial aggregates of two or more fronds in a colony. *Lemna minor* has single ovules and propagate vegatitivly and thus represent clones (Landolt, 1975). As *Lemna minor* mainly grows two-dimensionally on the water surface, the growth may not only be established on the basis of frond counting as suggested in the test guidelines. Alternatively, growth can be measured by determining the frond area.

The growth rate of these plants can be observed by frond counting or by measuring the frond area. The shape and frond size can be influenced by external factors (Landolt, 1975) such as toxic stress. As young fronds are small and may be overseen, the number of fronds counted depends on the experimenter. Additionally, the growth rate of the plant rather depends on the frond size than the frond number (Eberius et al., 2002) and thus the determination of the growth rate on the basis of the frond area should be preferred.

1.6 Possible variables influencing toxicity over time

1.6.1 The substances and their mechanism or mode of action

The Mode of Action (MoA) represents the cellular, physiological or organism-level effects of exposure to chemicals whereas the Mechanism of Action (MeoA) requires information on how a substance reacts on a molecular level with the target site. There is however no clear cut line between these two terms and contradictory definitions can be found (Escher and Hermens

2002). Recently Escher et al. (2011) classified the MeoA as the primary action of toxicant action on the molecular level which causes and is followed by the secondary action, the physiological effect or that is the MoA. Nevertheless, the differentiation between both terms remains ambiguous as the authors state. Concerning the introduced herbicides there is sufficient knowledge on how these substances react on a molecular level, hence their MeoA. Nevertheless the herbicides are grouped in accordance with the physiological responses they provoke i.e. their MoA. Concerning the metals there is however a lack of knowledge on how metals react on a molecular level in detail to provoke a particular effect. Therefore their introduction mainly focuses on physiological effects i.e. their MoA. If information on the molecular mechanism is available and this work uses the term MeoA otherwise the term MoA is used.

Main target sites of toxicants are membranes, proteins and genetic material (Escher and Hermens, 2002). The same chemical may operate by a number of MoAs. Different concentrations may have different or additional effects. Apart from specific MeoAs as introduced for the herbicides substances can also evolve unspecific effects. For instance, the interaction with membranes which is believed to cause narcosis, the so called base-line toxicity, is an intrinsic property of every chemical (Escher et al., 2006).

Herbicides

Herbicides acting on photosynthesis, PS2 system

Triazines

The s-triazines are used as active ingredients of herbicides and they are ubiquitous pollutants that are frequently found together or in close timely proximity in freshwater systems. Atrazine is the most frequently detected and most intensively studied representative of this group (Gfrerer et al., 2002; Garmouma et al., 2001; Clark and Goolsby, 2000; Müller et al., 2000; Thurman and Cromwell, 2000; Pempkowiak et al., 2000; Chevreuil et al., 1999; Clark et al., 1999). All herbicidal s-triazines target photosystem II where they compete with plastoquinone, the electron transporter at the reducing site. The N-3 of the triazine ring-system and the amino nitrogen at position 2 form hydrogen bonds to a peptide nitrogen and a serin residue. In this reversible procedure s-triazines can displace the natural electron acceptor plastoquinone, which causes an interruption of the electron transport (Mackay and O'Malley, 1993; Oettmeier, 1999; Oettmeier and Hilp, 1991). This electron transport is a light dependant procedure. Thus, this interruption has the same effect as if the plant were put into darkness. S-triazine exposure may also induce severe photo-oxidative stress, as the readily available

oxidative potential from excited pigments results in the production of Reactive Oxygen Species (ROS), which then irreversibly damage the proteins of the electron transport chain (Hock et al., 1995). Photooxidative stress can also occur under normal photosynthesis. Plants have thus developed defence mechanisms. The D1 protein, part of the electron transport chain, is especially vulnerable to photooxidation. Thus this protein has a rapid turnover rate. PSII inhibitors prohibit this turnover, which subsequently leads to irreversible photooxidative damage.

Diuron

Diuron is used as a total control of weeds and mosses on non-crop areas (Tomlin, 1994). Diuron belongs to the phenylurea. Like the triazines, Diuron acts on the photosynthetic system, interrupting the electron transport at the reducing site of PSII and inhibiting the turnover of the D1 protein. Thus, the effects of Diuron are the same as for the triazines. Diuron and the triazines have the equal binding niche, namely the binding site of the electrontransporter plastochinon PQB. However, triazines resistant mutants are nevertheless susceptible to Diuron which indicates a different binding. (Hock et al., 1995)

Herbicides acting on photosynthesis, PS1 system

Paraquat

Paraquat is used for control of broad-leaved weeds and grasses in a variety of different crops and for general weed control (Tomlin, 1994). Paraquat belongs to the bipyridines or so called „quats „due to the quaternary nitrogen. It is one of the early found herbicides in the fifties of the last century. Like Diuron and the triazines Paraquat acts on the photosynthetic system however at the PSI site at the exterior of the thylacoide-membrane as an electron-scavenger. Here the positively charged protein PsaD-Gens passes on two electrons to the iron-sulphur complex containing ferrodoxin. As Paraquat itself is positively charged, the electron transfer probably does not occur directly at the PsaD-Gens protein but from ferrodoxin (Bowyer and Camilleri, 1987). As Paraquat can only collect one electron, this substance thus converts into a radical with an unpaired electron, which is passed on to oxygen, which converts, into a superoxide-anion that finally evokes oxidative stress. As the superoxide-anion is relatively stable, it can diffuse into to other cell compartments. Hence, oxidative stress and damage does not necessarily occur in the photosynthetic compartment.

Herbicides acting outside the chloroplasts

Aclonifen

Aclonifen belongs to the nitrodiphenylethers and is used as pre-emergence control of grass and broad-leaved weeds especially in potatoes (Tomlin, 1994; Hock et al., 1995b). It blocks the protoporphyrinogen-oxidase (PPGO). Due to its structure with two aromatic rings and the position of the rings, it resembles one half of protoporphyrinogen IX (PPGIX). PPGIX is a precursor of porphyrin, which forms chlorophyll or cytochromes. Because of this resemblance, Aclonifen probably occupies one half of the binding site of PPGIX. Consequently, the enzyme PPGO is blocked which normally oxidises the PPGIX to protoporphyrin (PPIX). This oxidation can also occur spontaneously but under normal conditions this oxidation is controlled by PPGO. If however, this enzyme is blocked, as it is the case if Aclonifen is present, the uncontrolled formation of PPIX takes place. Like chlorophyll, this substance is activated by light and then transfers this activation energy onto oxygen, which converts into the harmful singulett oxygen. This may not only occur on chloroplasts but also in mitochondria, as the target enzyme exists in both organelles. (Hock et al., 1995)

Alachlor

Alachlor belongs to the diverse group of chloracetamides. The growth of annual grasses is especially effected by these herbicides (Tomlin, 1994). Although this substance class has been known for long time and has been thoroughly studied, the molecular MeoA is not fully understood. The effect on growth does not occur immediately. The cell growth as well as the cell division is affected by this substance class (Deal and Hess, 1980). Changes of the lipid composition and the gradual inhibition of lipid synthesis over time were observed on cells of kidney beans by Chang et al. (1985). This substance class has the property of alkylation (Fuerst, 1987). Thus, chloracetamides may react with the nucleophilic thiol-group (-SH) of proteins releasing chloride or an aryloxy residual. This hypothesis has been underpinned by Molin et al. (1990). Hence, the alkylation of enzyme-thiol groups and coenzyme A (CoA-SH) has been discussed as a MeoA.

Metals

Although some metals are essential nutrients, excess concentrations of all heavy metals lead to various toxic effects such as oxidative stress and inhibition of enzymes (Dietz et al., 1999; Pohlmeier, 1999). Copper and zinc belong to the essential metals. Nickel has been put forward as being essential for legumes (Ernst, 1998), and pecan (Wood et al., 2004). As no biological/biochemical functions are known for cadmium, it is characterised as non-essential.

According to the Pearson concept zinc, cadmium, nickel and copper belong to the so-called borderline group and are therefore able to form stable complexes with all categories of ligands such as oxygen, sulphur and nitrogen containing compounds like proteins (Nieboer and Richardson, 1980). As proteins are potential ligands for these metals, this property is utilized in the case of copper and zinc. These metals play vital roles in the physiology of the cell, e.g. as part of the prosthetic group in enzymes or they can structure proteins, e.g. in the so-called zinc-fingers of various DNA-binding proteins. Not designated chelatation with proteins may however lead to structure and functioning loss of enzymes.

Except for copper all metals chosen, show no redox-activity under physiological circumstances. Thus, copper can cause oxidative stress directly generating ROS. However many studies have shown that even the other metals cause lipid-peroxidation, induce enzymatic antioxidative responses and change the glutathione and ascorbate level indicating oxidative stress (Teisseire and Vernet, 2000; Gallego et al., 1999; Cuypers et al., 1999; Baccouch et al., 1998; Chaoui et al., 1997; Weckx and Clijsters, 1997; Gallego et al., 1996; Weckx Clijsters, 1996; Subhadra et al., 1991). The function loss of proteins might be one way oxidative stress is evoked.

1.6.2 Detoxification in plants

Pesticide biotransformation/detoxification

Plants have a general procedure for dealing with xenobiotics. The major route of detoxification is the oxidative, reductive or hydrolytic enzymatic transformation with the aim of producing functional groups in order to conjugate these groups with endogenous compounds such as glutathione, sugars or organic acids or to make them more vulnerable to further steps of metabolism (Hoagland et al., 2001b). Some of the enzymes are constitutively expressed whereas the formation of other enzymes needs to be induced (Hoagland et al, 2001b). Due to the broad substrate specifities of some enzymes, these enzymes detoxify endogenous as well as exogenous compounds. Thus, the modification of enzymes for pesticide metabolism is not necessary (Hoagland et al., 2001a) and pesticide resistance develops rather due to the modification of the target site than the modification of the metabolism (Saari, 1999). Esterase can also play a role in pesticide activation. Some herbicides have been specifically developed to exploit this metabolic pathway. The acid form of these pesticides is the active agent but as esters they are better absorbed by the plant.

Glucolysation and amino acid conjugation are dominant as glucose and amino acids are abundant in plants (Hall et al., 2001). Conjugation causes a higher molecular weight, enhanced water solubility, enhanced susceptibility to further transformation and decreased mobility of the compound. As plants lack excretory systems these modified compounds are generally compartmentalized into the vacuole the cell wall lignin or vascular tissue (Hall et al., 2001). For instance ATP-dependant pumps recognize the glutathione moiety and transport the glutathione-conjugates from the cytoplasm into the vacuole. For glucose or aminoacides conjugates such have not yet been discovered (Hall et al, 2001).

Metal detoxification

It is important for plants to acquire essential metals as micronutrients, but also to regulate their concentrations to prevent intoxication. Unlike pesticides metals are not vulnerable to transformation. Thus the strategy to overcome a metal intoxication or to control an excess metal concentration is immobilisation by means of chelatation to cystein-rich phytochelatines (Tsuji et al., 2002; Kwan and Smith, 1990a; Grill et al., 1987), sequestration in the vacuole and binding to organic acids (Krotz et al., 1989; Mathys, 1977) or reduction of the internal metal concentration via efflux (Hall and Williams, 2003; Williams et al., 2000). The formation of polypeptides is strongly induced by heavy metals and the strength of the induction depends on the metal species (Grill et al., 1985). These cystein rich polypeptides supply many thiol groups for binding metals but also prove themselves to be good antioxidants (Tsuji et al., 2002) and may be involved in the transport of cadmium into the vacuole (Vögeli-Lange and Wagner, 1990).

1.7 Research questions

Generally, toxicity studies focus on the question what concentration or dose is necessary to cause a certain effect (e.g. EC_{50}) or what concentration is tolerable for not causing an observable effect (e.g. NOEC). Toxicity studies generally focus on the dose of toxicant, testing various concentrations under continuous exposure conditions. Time is only regarded semi-quantitatively distinguishing between acute toxicity and chronic toxicity. However, time is equally important to the dose and only very few studies have investigated the relationship between toxicity and time so far. Studies that have been conducted are for example the works by Ashauer et al. (2006a, 2006b, 2007a, 2007b, 2007c), Hassold (2009) and Valloton (2008).

Time consists of many timescales, like toxicodynamic, the dynamic of injury and recovery and toxicokinetic, the dynamic of absorption and elimination of a toxicant. Are all timescales important? Studies often only consider toxicokinetics. However, toxicodynamics i.e. the

quality of the damage which is linked the MeoA and MoA may be an important factor that determines the toxicity-time-relationship. Therefore substances with different MeoA and MoA are investigated. Additionally, the uptake and elimination over time may determine the toxicity. As the internal metal concentration is easily measurable this issue has been investigated for the metals. Apart from the quality of the damage the quantity of damage may be also important. Different effect concentrations of the same substance may lead to different toxicity-time relationships. Therefore this work additionally analyses whether the toxicity-time relationships differ for different effect-concentrations.

There are different approaches, either empiric or mechanistic, which deal with the toxicity-time relationship. Originating from pharmacology, a simple empiric approach was developed at the beginning of the 20th century by Warren (1900) and modified by Ostwald and Dernoscheck (1910) and Bliss (1940). Haber (1924) simplified the equation even further in the 20s and also became its eponym, Haber's Law. It demands some experimental preconditions in order to observe Haber's Law (Rozmann, 2000). Whether these preconditions are met by the test used in this work and whether Haber's Law or its derivations are observable with the data achieved from this work will be investigated. DEBtox (Bedaux and Kooijman, 1994; Kooijman, 2000b) is a mechanistic model, which is based on assumptions concerning the factors that determine the toxicity-time-relationship. Whether these assumptions are sufficient, will be discussed on the basis of the findings of this work.

The exposure to pollutants is not necessarily continuous. A high concentration of a pollutant may be followed by a low concentration or no pollutant at all. This leads to complex exposure patterns. Aquatic organisms can therefore be exposed to single or repeated pulses of hazardous substances. If single pulses occur the organism may recover depending on the quality and quantity of the damage and the detoxification capability. The MoA and MeoA of a substance may give a conclusion on the recovery potential. If aquatic organisms are exposed to repeated pulses or fluctuating concentrations of a hazardous substance, different effects may be observed. The damage may cumulate, the organism may adapt or the history of exposure may have no impact at all. It may be possible to draw conclusions on the basis of investigated recovery potentials.

Apart from the exposure to single substances organisms in aquatic ecosystems are typically exposed to a multitude of pollutants. Considering different toxicity-time relationships for the different substances investigated the question arises how mixture toxicity changes over time if substances are combined. Additionally, mixtures are not necessarily of constant composition

as concerning quality and quantity. Different substances may occur in pulses at different time points. Can conclusions be drawn on the basis of the preceding studies of this work? So far the concepts CA and IA have proved to be good prediction tools for mixture toxicity for mixtures of constant qualitative and quantitative composition (Backhaus et al., 2004; Backhaus et al., 2003; Drost et al., 2003; Faust et al., 2003; Junghans et al., 2003; Faust et al., 2001; Altenburger et al., 2000; Backhaus et al., 2000; Hermens and Leeuwangh, 1982). If exposure occurs in fluctuating concentrations, organisms may be exposed to substances one at a time but nevertheless show combination effects. The extent to which mixture toxicity can be assessed by the concepts IA and CA in such scenarios is analysed.

Considering all the aspects mentioned above following major research questions will be investigated:

- If the exposure to a single hazardous substance is continuous, how does time determine toxicity and which factors are important for the toxicity-time-relationship?

- If the exposure to a single hazardous substance is not continuous but occurs in pulses or fluctuates; how does the toxicity change and what determines toxicity over time? Is there a recovery after a single pulse and what aspects determine the recovery potential?

- If the exposure occurs to mixtures under simple and complex exposure conditions, how does mixture toxicity change over time and is it predictable? What additional aspects may need to be taken into account if considering more complex exposure conditions.

2 Materials and methods

2.1 Technical Equipment

atomic adsorption spectrometer (AAS)	Perkin Elmer, PE 2880
automatic image evaluation system	LemanTEC Scanalyser**,** LemnaTEC GmbH
inductively-coupled-plasma optical-emission-spectrometer (ICP-OES)	Thermo-Electron Corporation, Trace Scan
graphite-tube atomic absorption spectrometer (AAS)	Thermo-Electron Corporation, Solar M
high performance liquid chromatograph (HPLC)	a Merck Hitachi chromatography system with: L-6200A intelligent pump AS-2000a autosampler Merck supersper RP18selctB 125/3,1 column L-4250 UV-VIS detector D-2300 chromato-integrator
flow injection analyser (FIA)	Foss Tecator, FIAstar
camera	Canon, PowershotA60
temperated chamber	Jürgens, Typ KLT 4
neon lights	Osram, 18W/25 universal white
six well plates	Greiner Biochemica
Erlenmeyer flasks	Schott Duran

Table 1: technical equipment used in this work

2.2 Test Substances

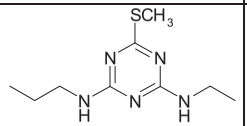
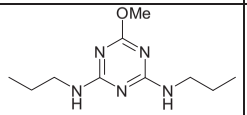
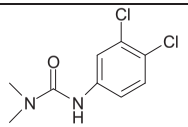
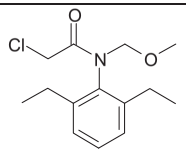
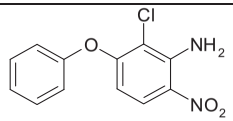
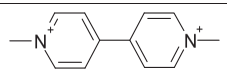
substance class	substance	CAS-Nr.	empirical formula	structural formula	molecular weight [g/Mol]	purity [%]	acquired from
triazine	Ametryn	834-12-8	$C_9H_{17}N_5S$		227,33	98	Riedel de Haën
triazine	Prometon	1610-18-0	$C_{10}H_{19}N_5O$		225,29	99	Riedel de Haën
phenylurea	Diuron	330-54-1	$C_9H_{10}Cl_2N_2O$		233,10	pestanal	Riedel de Haën
chloracetanilide	Alachlor	15972-60-8	$C_{14}H_{20}ClNO_2$		269,8	99,7	Riedel de Haën
Nitrodiphenyl-ether	Aclonifen	74070-46-5	$C_{12}H_9ClN_2O_3$		264,67	99	Riedel de Haën
bipyridine	Paraquat dichloride	1910-42-5	$C_{12}H_{14}N_2^{+2}+2Cl^-$			99	Riedel de Haën
metal	Zinc chloride	7646-85-7	$ZnCl_2$		136,28	99	Merck
metal	Copper chloride	7447-39-4	$CuCl_2\ 2H_2O$		134,45	97	Merck
metal	Nickel sulphate	10101-97-0	$NiSO_4\ 6H_2O$		262,82	99	Merck
metal	Cadmium sulphate	7790-84-3	$CdSO_4\ 6H_2O$		769,51	98	Merck

Table 2: Test substances. The substances were analysed in the growth inhibition test with *Lemna minor*. All other chemicals were obtained from standard providers in p.a. quality

2.3 Cultivation of the duckweed

Initial plant material was kindly provided by Dr. Rolf Altenburger (Helmholtz Zentrum Umweltforschung Leipzig-Halle, Germany) and by Dr. Nina Cedergreen (Royal Veterinary and Agricultural University (KVL), Frederiksberg, Denmark).

Lemna minor was cultivated according to DIN AK, 2001. The plants were grown in sterilised Steinberg medium (3,46 mM KNO3, 1,25 mM $Ca(NO_3)_2$, 0,66 mM KH_2PO_4, 0,072 mM K_2HPO_4, 0,41 mM $MgSO_4$, 1,94 µM H_3BO_3, 0,63 µM $ZnSO_4$, 0,18 µM Na_2MoO_4, 0,91 µM $MnCl_2$, 2,81 µM $FeCl_3$, 4,03 µM EDTA ;pH 5,5+/-0,2) in a temperated chamber (Jürgens, Typ KLT 4, 25 +/-2°C). The chamber was continuously illuminated (Osram, 18W/25, universal-white, 85-125 µE/m² x sec). With the exception of the chamber temperature which was modified to 24 +/-2°C these test conditions are identical to the DIN and ISO standard finalized in 2006 (International Organization for Standardization (ISO), 2006;Organisation for Economic Co-operation and Development (OECD), 2006).

In order to conserve the original defined *Lemna*, the plants were kept as a stock culture on agar consisting of 1,5 % agar containing Steinberg medium. The stock culture was kept sterile in an Erlenmeyer flask which was closed with a cotton plug. The flasks were stored on a shelf with normal daylight and day and night rhythm.

Lemnas used for testing were pre-cultured prior to use. The plants were then grown in normal Steinberg medium in a temperated chamber with continuous light as described above in open Erlenmeyer flasks. For testing the growth rate of the test plants had a doubling time of 2,5 days and the plants had young, rapidly growing colonies with bright green colour without visible lesions, chlorosis or necrosis. The colonies chosen for testing consisted of three or four fronds.

2.4 Estimation of the growth rate

Experiments with the triazines and the heavy metals were conducted using the frond number as the endpoint. During this work, a new method to determine the growth rate was established and thus the for experiments with the herbicides Alachlor, Aclonifen, Diuron and Paraquat the total area of the plant was used as an indicator of growth.

2.4.1 Growth pattern

As exemplified with *Lemna minor* exposed to Ametryn (Figure 1) and Paraquat (Figure 2) shown in the growth pattern of untreated *Lemna minor* and *Lemna minor* during exposure was

exponential in all cases. An exposure led to a concentration-dependent reduction in the growth rate, while the growth pattern itself was unchanged. There was no difference whether growth was measured via frond number or the whole area of the plant. Stagnancy in growth was not observed (Figure 1 and Figure 2).

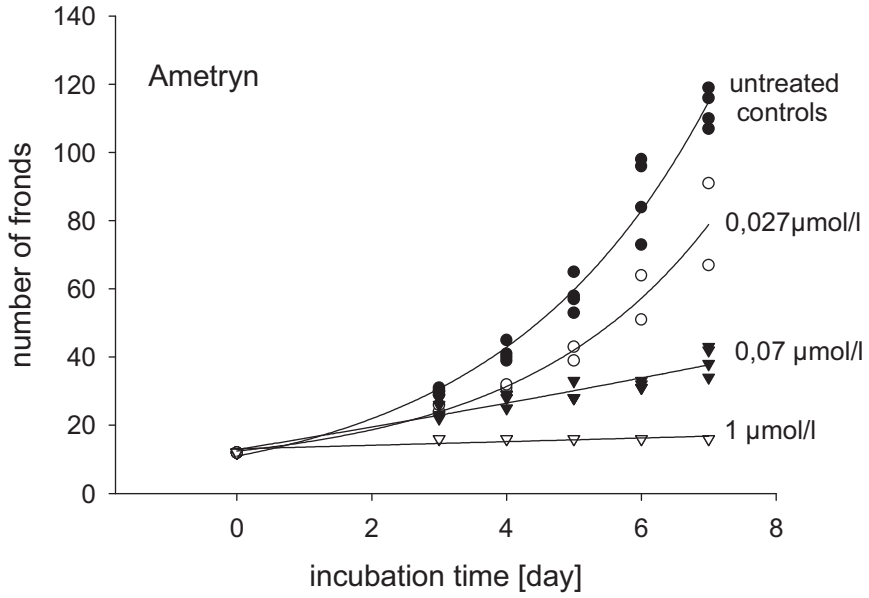


Figure 1*: Exponential growth pattern of *Lemna minor* based on frond number. Concentrations refer to incubation with Ametryn

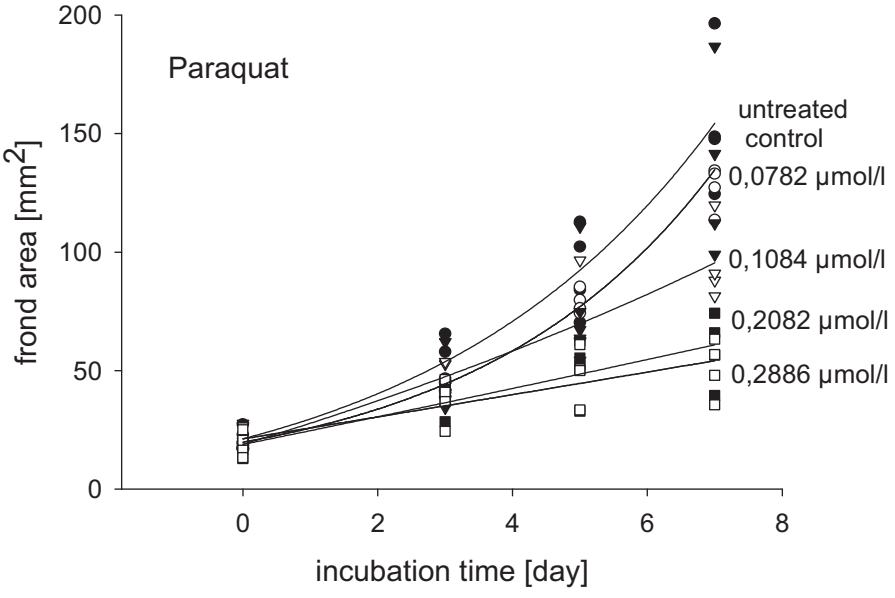


Figure 2: Exponential growth pattern of *Lemna minor* based on frond area. Concentrations refer to incubation with Paraquat.

∗ figure 1 has already been published elsewhere; see Drost W, Backhaus T, Vassilakaki M, Grimme LH (2003): Mixture of s-triazines toxicity to Lemna minor under conditions of simultaneous and sequential exposure. Fresenius Environmental Bulletin 12(6): 601-607

2.4.2 Estimation of the growth rate via frond counting

Tests based on frond number were conducted in Erlenmeyer flasks (Schott, Duran glass). According to the DIN AK, 2000 the test starts with 12 fronds and the endpoint is the growth rate at day seven. However, in order to detect a time dependence of toxicity of a substance, the test was modified and counting of the fronds was started at the third day of testing. Furthermore, the test was started with 24 fronds instead of the recommended 12. Tests conducted with initial 12 fronds led to scattered plots and as this leads to a less precise determination of the effect concentration values at day three the initial frond number was thus doubled. The fronds were counted every 24 hours starting at day three.

2.4.3 Estimation of the growth rate via frond area

Test plants were taken from a pre culture as described above. Instead of Erlenmeyer flasks the test plants were placed in six well plates with lids (Greiner Biochemical). Each well was filled with 10 mL solution. Four wells of each plate were used for testing the concentration response relationship of a substance whereas two wells were used for control plants. Each well was equipped with one or two colonies with three or four fronds. The area of the fronds was established by means of photography. The camera was adjusted parallel to the plant area or the six well respectively and the whole plate was photographed. The frond area was analysed with Photoshop (Vers. 7.0, Adobe Systems, San Jose, USA) by establishing the pixel number of the assigned area. The pixel number of the fronds was referred to a so called „dummy *Lemna*“ with a well-known mm² area which was added to the six well plates when photographed and which was also analysed with Photoshop.

2.4.4 Determination of the endpoint

For all tests the growth rate μ was used as the endpoint which is calculated on the basis of the number of fronds:

$$\mu = \frac{\ln(F_{t_2}) - \ln(F_{t_1})}{t_2 - t_1} \qquad (1)$$

or if the growth rate was based on the frond area:

$$\mu = \frac{\ln(A_{t_2}) - \ln(A_{t_1})}{t_2 - t_1} \qquad (2)$$

F_{t1} and F_{t2} are the frond numbers at day t_1 and day t_2 of the experiment, respectively. μ is based on the assumption of an exponential growth and gives an average of the growth during

the time period from the start of the experiment to day t_2. The growth rate based on frond area was calculated likewise. Growth inhibition was calculated in the following way:

$$\%inhibition = 100 \times \left(1 - \frac{\mu_{sample}}{\overline{\mu}_{control}} \right) \qquad (3)$$

2.4.5 Preparation of the test solutions

Herbicides

The herbicides investigated were purchased in the highest available purity from Riedel de Haën (Seelze, Germany) or Merck (Darmstadt, Germany). From each compound a stock solution in methanol (p.a.) was prepared and stored at -20°C. Prior to testing an aqueous solution was prepared from these stocks by evaporating the methanol under a gentle stream of N_2 and adding the necessary volume of bi-distilled water. This solution was kept strongly stirred in the dark for at least 24 hours prior to testing to allow the test compound to dissolve completely. Subsequently the dilution to obtain the investigated concentrations of the test substances was carried out. Afterwards, the growth medium was added to the dilutions in a twofold concentration. Except for Paraquat the stability of the tested herbicides over an incubation period of 7 days was checked via HPLC. All test substances proved to be stable within +/- 10% of the starting concentration.

Metals

The four metals tested (copper, zinc, nickel and cadmium) were used in the highest available purity and were purchased from Riedel de Haën (Seelze, Germany) and Merck (Darmstadt, Germany). Zinc and copper were used as chlorides, nickel and cadmium as sulphates. Aqueous stock solutions in bi-distilled water were prepared and test solutions were made taking the necessary aliquots from the stock solutions. The growth medium in a twofold concentration was added after preparing the various test concentrations. The concentrations of the heavy metals were checked via AAS and proved to be constant over time.

2.5 Test procedures

2.5.1 Standard toxicity test

Toxicity studies were conducted in accordance with the guidelines ((International Organization for Standardization (ISO), 2006; Organisation for Economic Co-operation and Development (OECD), 2006). For the determination of concentration-response curves the plants were exposed to a geometric concentration series of each test substance. At least five

concentrations, covering 0-100% effect were tested in triplicates (frond counting) or quadruples (frond area). For all tested substances complete concentration response curves were recorded after the standard exposure duration of seven days as well as after an exposure of three, five and six days. For data based on frond number, complete concentration response curves were also recorded at day four.

2.5.2 Recovery experiments

In the case of recovery experiments the plants were exposed three days to the previously determined ECxd3 concentrations. The fronds from each treatment were counted or the frond area was acquired for the determination of the growth rate over the first three days. Fronds of each sample were subsequently transferred into fresh nutrient medium restarting with 24 fronds if the growth rate was established by means of frond counting. The recovery was then recorded for another seven days observing the growth rate.

2.5.3 Sequential exposure

In order to interpret the influence of a pre-exposure on the concentration response relationship of a second substance, the substances were combined using a fixed level of the first substance combined with varying concentrations of the second substance. This means, the plants were pre-treated in the same manner as in the recovery experiments but the plants were subsequently exposed to a second substance instead.

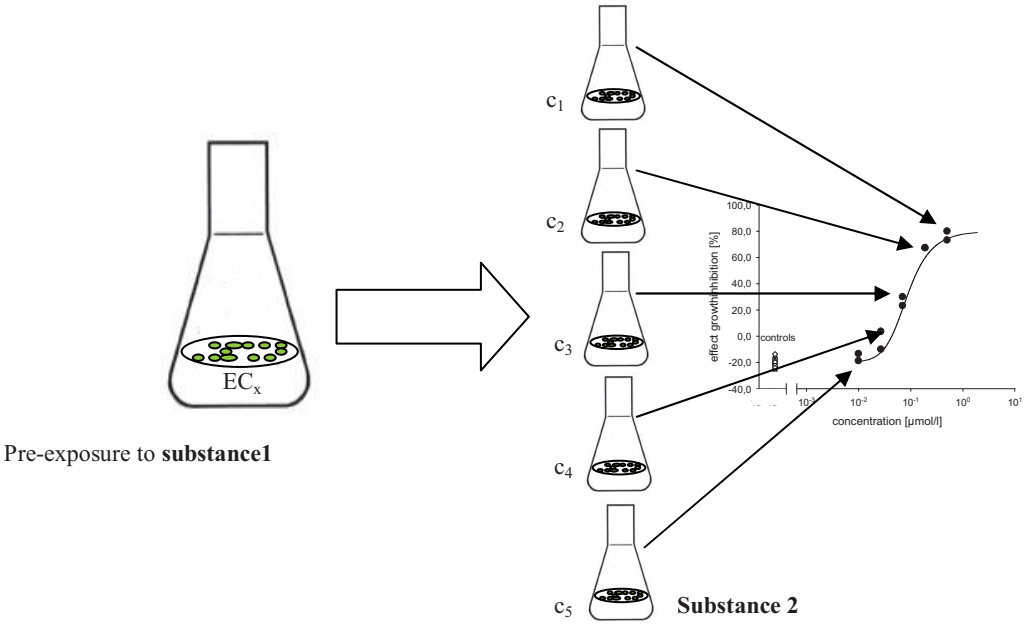


Figure 3: scheme of a sequential exposure: pre-exposure to a single concentration of substance 1, subsequent exposure to different concentrations of substance 2

2.5.4 Mixture toxicity experiments

Mixture experiments were designed on the basis of the predictions made by CA and the substances were mixed in the relation of their EC_{50} ratios a fixed ratio. The predictions were calculated using the fits of the single concentration response curves. The total concentration of the mixture was systematically varied. At least five concentrations, covering the assumed 0-100% effects predicted by CA were tested in triplicates (frond counting) or quadruples (frond area).

2.6 Calculations

2.6.1 Concentration response modelling

For the determination of concentration-response curves the plants were exposed to a geometric concentration series of each test substance. At least five concentrations, covering 0-100% effect were tested in triplicates (frond counting) or quadruples (frond area). The concentrations given in the concentration response curves are the nominal concentrations. The dilution factor was dynamically adjusted to the steepness of the concentration-response curves as determined from previous range-finding experiments. For the determination of concentration-response curves the plants were exposed to a geometric concentration series of each test substance. Biometrical concentration response modelling was carried out with SAS (Vers. 8.2, SAS-Institute, Cary, USA) or Sigma Plot (Vers. 10.0, Sysdat Software Inc.,) using one of the following three-parametric generalized logit models (Scholze et al., 2000).

$$Eff = \frac{1}{\left(1+\exp(-\theta_1 - \theta_2 \log_{10}(conc))\right)^{\theta_3}} \qquad \text{(Generalised Logit 1)}$$

and

$$Eff = 1 - \frac{1}{\left(1+\exp(\theta_1 + \theta_2 \log_{10}(conc))\right)^{\theta_3}} \qquad \text{(Generalised Logit 2)}$$

or four parametric Weibull fit

$$Eff = \frac{\theta_1}{1-\exp(c - c_0 + \theta_2 \ln 2^{1/\theta_3})/\theta_2}$$

where $\theta_1, \theta_2, \theta_3$ are the three parameters to be estimated. 95% approximate confidence limits were calculated using the standard Wald-based approach of SAS *proc nlin*. (Statistical significances were calculated using the standard two-sided *t*-test (α=0.95)).

2.6.2 Calculation of the mixtures

Simultaneous mixture experiments were designed on the basis of the predictions made by Concentration Addition (CA). The predictions were based on the single substance concentration-response curves. As the mixture components were set to be present in a constant proportion *p* in the mixture ($p_i=c_i/c_{mix}$), the individual concentrations c_i of each component provoking x% effect in the mixture can be calculated as

$$c_i = p_i ECx_{mix} \qquad (4)$$

and the formula for CA as presented in chapter 1 is rewritten as

$$ECx_{mix} = \left(\sum_{i=1}^{n} \frac{p_i}{ECx_i} \right)^{-1} \qquad (5)$$

Hence, the effect concentrations for the mixture as predicted by CA were calculated based on the respective proportions and on the basis of the single substance effect concentrations. Using eq. (5) the total concentrations of each mixture giving 1-99% effect were calculated in steps of 1%. The resulting 99 concentration/effect pairs were plotted to obtain complete concentration response curves.

In contrast to CA, which is based on effect concentrations, Independent Action(IA) combines independent probabilities of an event and can only be calculated from effects. To calculate mixture effects as predicted by IA for complete concentration response curves, the concentration-response relationship obtained for CA was used as a starting point. For each effect concentration, previously calculated for CA, the concentration of each individual component c_i present in the mixture was calculated as $c_i=p_iECx_{mix}$. Based on the re-arranged regression models and parameter estimates describing the concentration response relationships of the single substances, the corresponding individual effects $E(c_i)$ for each substance were subsequently calculated at each concentration. Hence, using these single effects, the predicted mixture effect $E(c_{mix})$ was calculated according to Independent Action, where c_{mix} is the sum of all single concentrations c_i.

$$E(c_{mix}) = 1 - \prod_{i=1}^{n} (1 - E(c_i)) \qquad (6)$$

Again all concentration-effect pairs were plotted to obtain the predicted curve.

Combination effects caused by a sequential exposure to different substances were also predicted with the two concepts CA and IA. Though the plants were not exposed to mixtures but to single substances sequentially the concepts CA and IA were applied as a mixture may occur in the organism. Opposite to the simultaneous exposure to different substances the experiment was not designed on basis of the prediction but the predictions were made on the basis of the experiments, which means in the case of CA the effects were calculated to the given concentrations iteratively.

2.7 Chemical analysis

2.7.1 High Liquid Chromatography (HPLC)

Samples of the test solutions were taken at the beginning and ending of the experiment in order to control the concentration and stability of the substances. The analysis was conducted with a reversed phase column (Merck Supersper RP18selectB 125/3.1) and a bi-distilled water and acetonitril mixture. The substances were detected with a UV-VIS detector (Merck Hitachi L-4250 UV-VIS).

substance	**mobile phase**	**detection wavelength [nm]**	**injection volume [µl]**	**flow rate [ml/min]**
Aclonifen	50% 0,01M phosphate buffer 50% acetonitril	238	40	1
Alachlor	50% bidest 50% acetonitril	215	50	1
Ametryn	50% amoniumacetate 50% acetonitril	220	20	1
Diuron	50% 0,01M phosphate buffer 50% acetonitril	215	40	1
Prometon	50% amoniumacetate 50% acetonitril	220	20	1

Table 3: analytical settings for the detection and quantification of the test substances

2.7.2 Atomic Absorption Spectroscopy (AAS)

Samples of the metal test solutions were taken at the beginning and ending of the experiments to control the concentrations. In order to determine internal metal concentrations, the fronds

from each sample were dried to complete dryness at 60 C° and weighed. They were then digested in 4 ml of a 1:1 solution of 30% H_2O_2 and 65% HNO_3 in plastic tubes at 80°C. Afterwards, each sample was dried and then re-dissolved in 1 ml 0.3% HNO_3. Metal concentrations were determined using a Trace Scan inductively-coupled-plasma optical-emission-spectrometer (ICP-OES) for zinc and copper and a Solar M graphite-tube atomic absorption spectrometer (AAS) for nickel and cadmium (both Thermo-Electron Corporation, Dreieich, Germany). The analysis was performed by a contract laboratory (Medizinisches Labor Bremen, Haferwende 12, 28357 Bremen). For copper and zinc mixture experiments the metal concentration in the plants were detected with a Perkin Elmer 2880 atomic-absorption spectrometer.

Statistical significances of the metal concentrations in the different plant samples were calculated using the standard two-sided *t*-test (α=0.95).

2.7.3 Flow Injection Analysis (FIA)

To assure that the zinc toxicity was not caused by a lack of phosphate due to precipitation as zinc phosphate, the total phosphorus content of the growth medium was measured colormetrically as a yellow phosphomolybdic acid via flow injection analysis (Fiastar, Foss Tecator, Denmark). The isopolyacid is transferred into the phosphorus containing heteropolyacid. The oxidation state of both elements phosphorus and molybdate remains unchanged.

$$12(NH_4)_6Mo_7O_{24}+H_3PO_4+72H_3O^+ \rightarrow 7H_3[P(Mo_3O_{10})_4]+72NH_4^+ +108H_2O$$

3 Results

3.1 Proceeding

This work investigates with the toxicity of herbicidal acting compounds and heavy metals on *Lemna minor*. The tested substances were categorized into herbicides with different modes of action (Aclonifen, Alachlor, Ametryn, Diuron, and Paraquat), the triazines Ametryn and Prometon, and heavy metals (copper, zinc, nickel and cadmium). In accordance with the categorization of the substances, the results are divided into two groups, the herbicides and the heavy metals. The results are presented in accordance with the questioning of this work. The results are presented in the following order starting with single substance toxicity over time observed for continuous exposure regimes, followed by the observed toxicity and the observed recovery potential if exposure to single substances occurs in pulses, and closes with observed combination effects of mixtures of substances occurring simultaneously and in a constant composition and the combination of substances occurring sequentially. The data of the triazines as well as the heavy metals were based on the frond number whereas the data of the remaining herbicides were based on frond area.

3.2 Continuous exposure to single substances

3.2.1 Visually recorded effects

Herbicides

Due to the different MoA of the investigated herbicides, a variability of effects could be seen on *Lemna minor*. With the exception Paraquat, morphological changes of *Lemna minor* became especially visible at high effect concentrations. Therefore in order to illustrate the effects pictures were chosen, where the plants had been exposed to the respective highest applied concentration in the test as shown in Figure 4. All pictures were taken after a seven-day exposure. In the case of Aclonifen exposed plants daughter fronds showed a loss of pigments leading to pale green fronds. Both concentrations of Aclonifen (0,31µmol/l and 5µmol/l) caused the same level of growth inhibition, nevertheless there was a clear difference when comparing the degree of pigment loss. Alachlor exposed plants showed a normal green colour but dwarfish daughter fronds. Plants appeared normal if exposed to PSII inhibitors such as Diuron and Ametryn. They showed normal healthy green fronds but the colonies disintegrated at high effect concentrations. Necrosis and chlorosis was recorded for Paraquat-exposed plants leading to speckled green and white fronds or totally necrotic white fronds at high effect concentrations.

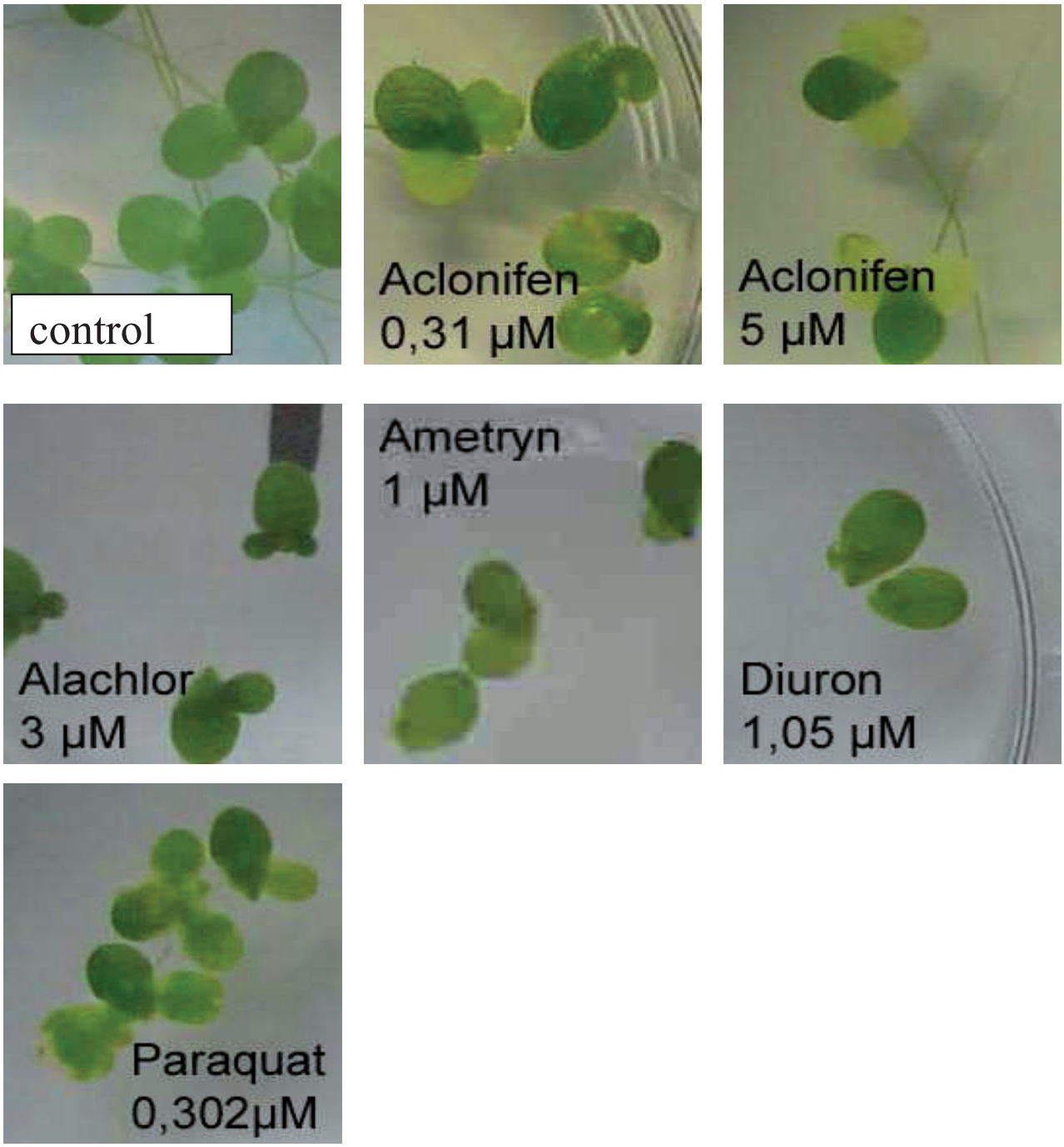


Figure 4: visually recordable effects of herbicides on *Lemna minor:*
All pictures were taken after a seven day exposure. The pictures are enlarged in order to better illustrate the visually recordable effects. The exposure concentration is indicated in the picture

Metals

The photographs of cadmium and nickel and exposed *Lemna* were taken by means of LemnaTec with a light source from under the plants, whereas normal photography with daylight was utilized for copper and zinc exposed plants (Figure 5). All indicated concentrations had an effect of approximately 50% growth inhibition and the pictures were taken after a seven-day exposure. In general all plants were smaller than the control plants and showed concentration-dependent chlorosis and necrosis. Nickel contaminated plants had chlorotic daughter fronds whereas in all other cases chlorosis started from the tip of the mother fronds. Due to the rising toxicity of zinc over time zinc, exposed plants were severely damaged after seven days with white necrotic fronds and disintegrated colonies.

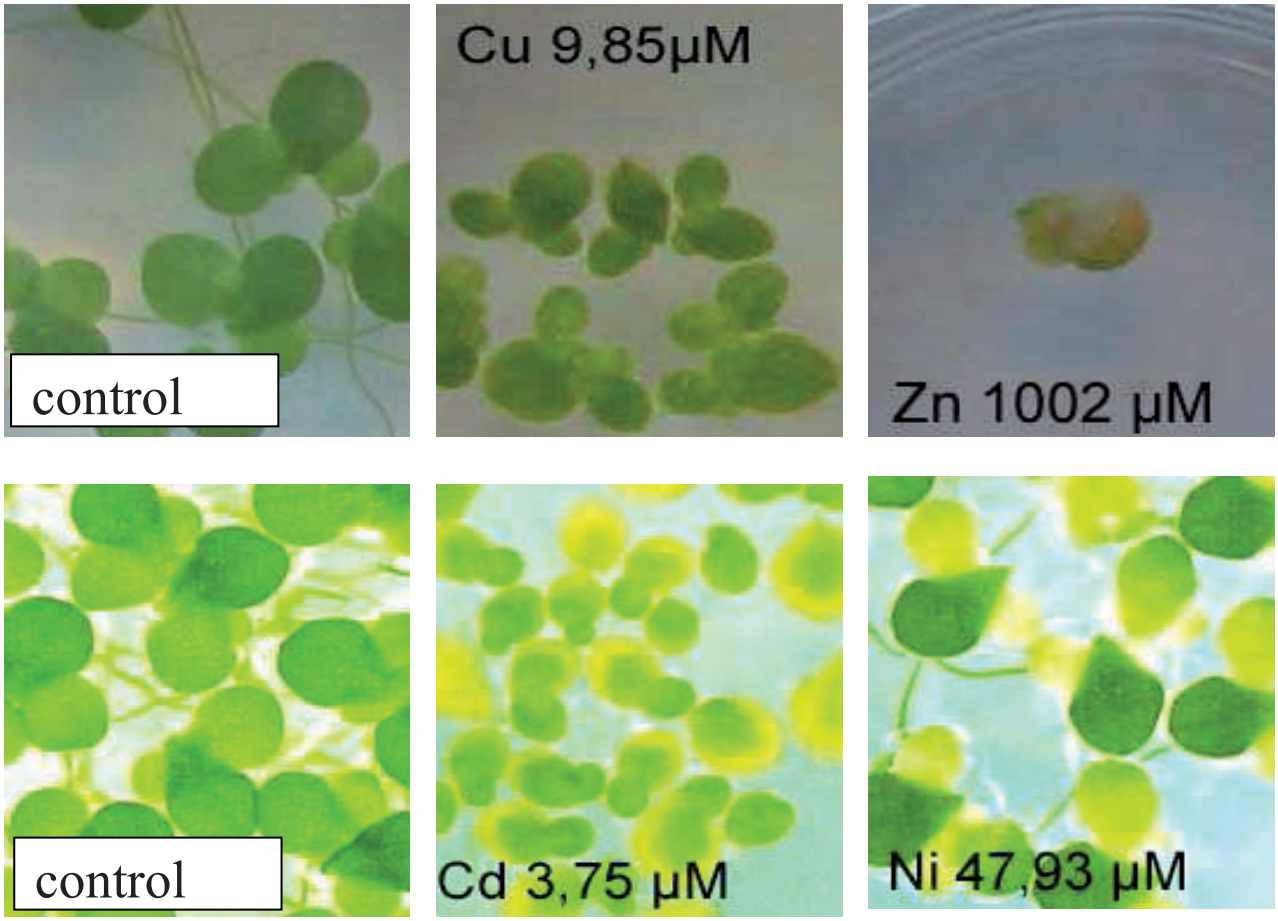


Figure 5: visually recordable effects of metals on *Lemna minor:*
All pictures were taken after a seven day exposure. The pictures are enlarged in order to better illustrate the visually recordable effects. The exposure concentration is indicated in the picture.

3.2.2 Observed toxicity-concentration response relation over time

Herbicides

	Ametryn			Prometon		
day	EC_{50}	$EC_{50(day\ 3)}$ /$EC_{50(day\ x)}$	EC_{75}/ EC_{25}	EC_{50}	$EC_{50(day\ 3)}$ /$EC_{50(day\ x)}$	EC_{75}/ EC_{25}
3	0,117		3,82	2,563		3,30
4	0,104	1,13	3,67	2,700	0,95	3,39
5	0,088	1,34	3,84	2,600	0,99	3,33
6	0,083	1,41	3,50	2,666	0,96	3,72

Table 4: growth inhibition of herbicides based on frond number:
The EC_{50} values at different days of the experiment are displayed for comparison. The EC_{50} values are given in µmol/l. The $EC_{50(day3)}$ /$EC_{50(day\ x}$)ratio provides and estimates the dynamics of toxicity for different exposure times. The EC_{75}/EC_{25} ratio of the triazines gives an indication of the average steepness of the curve in the middle effect region

For all tested substances complete concentration response curves were recorded after the standard exposure duration of seven days as well as after an exposure of three, five and six days. For data based on frond number, which is the case for the triazines, complete concentration response curves were also recorded at day four. (see annex Figures 35A to 42A)

Ametryn was the most toxic herbicide with an EC_{50} of 0,085 µmol/l at day seven, whereas Prometon was the least toxic with an EC_{50} of 2,797 µmol/l (Table 4). Both are PSII inhibitors and belong to the same chemical substance class, the triazines.

All other herbicides had toxicity within this range. Alachlor was similarly toxic to Ametryn with an EC_{50} of 0,097µmol/l but an effect of 100 percent growth inhibition could not be achieved if experiments were conducted in accordance with DIN 2001. This is similar in the case of Aclonifen, which was the third most toxic substance with 0,135 µmol/l, but the maximum effect was at approximately 60%. Diuron, also belonging to the PSII inhibitors but a phenylurea, was the second toxic PSII inhibitor with an EC_{50} of 0,152µmol/l. (Table5)

There was a slight increase in toxicity over time in the case of Ametryn and a slight decrease in toxicity in the case of Prometon. The calculated $EC_{50(day3)}$/$EC_{50(dayx)}$ ratio estimated the change of toxicity from day three to day x. The maximum factor was 1,41 for Ametryn

between day three and six, but generally the factor was close to one and thus the changes in toxicity over time were small (Table 4). The calculated EC_{75}/EC_{25} ratio of the triazines gives an indication of the average steepness of the curve in the middle effect region. Like the EC values the steepness of the concentration response curves of the triazines remained fairly unchanged over time and the ratio was roughly three (Table 4).

There was a clear enhancement of toxicity for Alachlor, Aclonifen and Paraquat as indicated by the $EC_{50(day\ 3)}$ / $EC_{50(day\ x)}$ ratio which increased over time. For Aclonifen and Paraquat an approximate twofold increase of toxicity was observed, whereas the toxicity of Alachlor rose by a factor of about three (Table5). The changes in toxicity were especially detected in the first half of the experiment. Comparing the EC_{50} values of day six and day seven, the EC_{50} values were nearly unchanged. The toxicity of Diuron did not show any tendency (Table5).

With the exception of Alachlor and Diuron, the steepness of the concentration response varied only little over time. Generally, there was a slight decrease of the EC_{25}/EC_{75} ratios meaning an increase of steepness. The EC_{25}/EC_{75} ratios of Alachlor declined from 141 to 6,8 (Table5). This is due to the temporal enhanced toxicity at higher concentrations. The change of steepness was not as drastic for Diuron changing from an EC_{25}/EC_{75} ratio of 7,98 at day three to 3,20. For a detailed comparison of the change of the concentration response curves over time the concentration response curves of all tested substance are included in the annex (annex Figures A48-A53).

Table 5: growth inhibition of herbicides based on frond area:
The EC_{50} values at different days of the experiment are displayed for comparison. The EC_{50} values are given in µmol/l. The $EC_{50(day3)}$ / $EC_{50(day\ x)}$ ratio provides and estimates the dynamics of toxicity for different exposure times. The EC_{75}/EC_{25} ratio gives an indication of the average steepness of the curve in the middle effect region.

day	Aclonifen			Alachlor			Diuron			Paraquat		
	EC_{50}	$EC_{50(day\ 3)}$ /$EC_{50(day\ x)}$	EC_{75} / EC_{25}	EC_{50}	$EC_{50(day\ 3)}$ /$EC_{50(day\ x)}$	EC_{75} / EC_{25}	EC_{50}	$EC_{50(day\ 3)}$ /$EC_{50(day\ x)}$	EC_{75} / EC_{25}	EC_{50}	$EC_{50(day\ 3)}$ /$EC_{50(day\ x)}$	EC_{75} / EC_{25}
3	0,285		2,63	0,314		141,56	0,124		7,98	0,549		4,50
5	0,157	1,82	1,98	0,179	1,75	62,74	0,202	0,61	6,01	0,321	1,71	4,22
6	0,088	3,24	2,26	0,129	2,43	6,80	0,111	1,12	4,81	0,319	1,72	3,45
7	0,097	2,95	2,21	0,135	2,33	6,81	0,152	0,82	3,20	0,294	1,87	3,30

Metals*

For all tested heavy metals complete concentration response curves were recorded after the standard exposure duration of seven days as well as after an exposure of three, four, five and six days. The growth was established on the basis of the frond number (see annex Figures 43A to 47A). Shown in Table 6are the EC values obtained according to the standard test procedure after seven days.

The nonessential heavy metal cadmium was the most toxic with an EC_{50} of 1,895 µmol/l. The essential metals copper, zinc and nickel showed considerably lower toxicities with EC_{50} values ranging between 9,682 and 56,283 µmol/l. (Table 6)

The toxicity of nickel and copper increased only slightly. The ratio between $EC_{50(day\ 3)}$ and $EC_{50(day\ x)}$ was higher than one, but was always lower than 1,5 (Table 6). The toxicity of cadmium also increased slightly, the EC_{50} reduced from 3,5 µmol/l after three days exposure to 1.9 µmol/l after seven days. In contrast to all other tested metals, the toxicity of Zinc increased drastically by a factor of more than 20 (Table 6). After nine days of exposure, the observed toxicity was even nearly 40 times higher than after three days. The high dynamic of Zinc toxicity could be directly observed by the unaided eye. After three days exposure, the *Lemnas* showed a reduced growth rate but were still green, while after seven days severe chlorosis was noted (Figure 5).

Zinc was the least toxic of the metals tested on day three, but there was a clear shift of the concentration response curve to lower concentrations over time (annex Figure 27A). This led to the effect that Zinc became similarly toxic to nickel with an EC_{50} value of 43 µmol/l and nickel with an EC_{50} value of 56 µmol/l (Table 6). Given in parenthesis are EC_{50} values for copper which were obtained approximately two years after the first experiments. Here the plants were about ten times more sensitive to copper than two years before though the culture and test procedure was not changed. For a detailed comparison of the change of the concentration response curves over time the concentration response curves of all tested substance are included in the annex (annex Figure 54A-57A).

* Parts of this chapter and all tables and figures have already been published elsewhere; see: Drost W, Matzke M, Backhaus T (2007): Heavy metal toxicity to Lemna minor: studies on the time-dependence of growth inhibition and the recovery after exposure. Chemosphere 67(1): 36-43

Table 6: growth inhibition of metals based on frond number:
The EC_{50} values at different days of the experiment are displayed for comparison. The EC_{50} values are given in µmol/l. The $EC_{50(day3)}/EC_{50(day\ x)}$ ratio provides an estimation of the dynamics of toxicity for different exposure times. EC_{75}/EC_{25} gives an indication of the average steepness of the curve in the middle effect region.

day	Zn			Cu			Ni			Cd		
	EC_{50}	$EC_{50(day\ 3)}/EC_{50(day\ x)}$	EC_{75}/EC_{25}	EC_{50}	$EC_{50(day\ 3)}/EC_{50(day\ x)}$	EC_{75}/EC_{25}	EC_{50}	$EC_{50(day\ 3)}/EC_{50(day\ x)}$	EC_{75}/EC_{25}	EC_{50}	$EC_{50(day\ 3)}/EC_{50(day\ x)}$	EC_{75}/EC_{25}
3	1045,256		46,53	12,140 (1,746)		2,30	81,778		3,05	3,526		19,58
4	510,052	2,05	61,00	11,147	1,09	2,44	70,316	1,16	4,54	3,035	0,99	23,42
5	301,354	3,47	148,03	9,881	1,23	2,47	62,476	1,31	4,79	2,754	1,11	20,83
6	221,494	4,72	174,49	9,648 (1,789)	1,26	2,36	56,941	1,44	5,40	1,855	1,16	32,56
7	43,048	24,28	360,92	9,682	1,25	2,39	56,283	1,45	5,49	1,895	1,15	21,00
8	32,441	32,22										
9	26,541	39,38										

Internal concentration over time

	Conc. in untreated controls (nmol/mg dry weight)	EC_{50} (µmol/l) (external)	EC_{50} (nmol/mg dry weight (internal)	BCF
Cd	0,0137 ± 0.016	3,7	4,8	1371
Cu	0,0654 ± 0.1	14,8	6,3	521
Ni	<0,4	78,0	5,3	65
Zn	3,5 ±1	1037,3	105,2	102

Table 7: internal concentrations of the tested metals in untreated controls and average bioconcentration factors at EC_{50}

The internal concentrations of zinc, copper, nickel and cadmium were measured using Atomic Absorption Spectroscopy (AAS) and the bioconcentration factor (BCF) for each metal was calculated as the ratio of internal to external EC_{50} concentration (Table 7). Zinc and copper being essential metals for plants were also found in the untreated controls. Traces of cadmium were also found in the control (Table 7), although the growth medium was prepared using salts of the highest purity available. Nevertheless, compared to the concentrations detected in the exposed plants, the metal levels in the control plants were negligible.

The internal concentrations of copper, nickel and cadmium that caused a growth reduction of 50% were similar, despite their largely differing external EC_{50} values (Table 6). Cadmium had the highest BCF of more than 1300. For zinc a bioconcentration factor of 102 was determined. This metal showed the lowest toxicity to the growth of *Lemna minor*, no matter whether based on external or internal concentrations.

Internal metal concentrations varied slightly over the exposure duration of 3-7 days, but no strong, consistent pattern was found (Figure 6). For zinc and cadmium slight but significant decreases of the internal concentrations were detected.

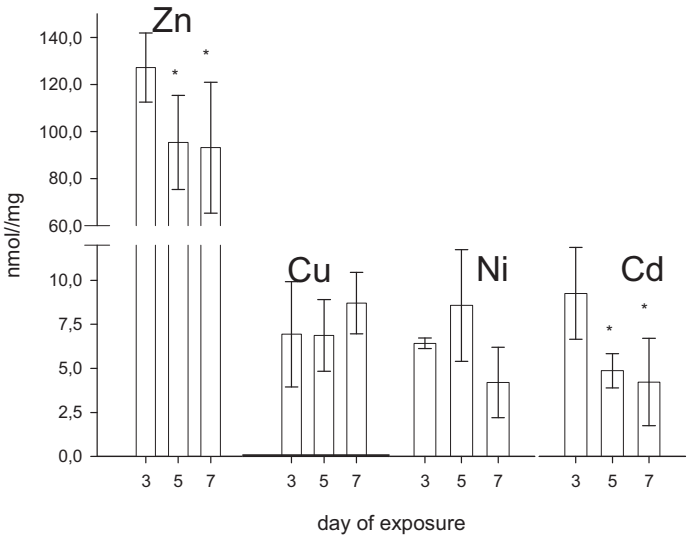


Figure 6: Internal metal concentrations in *Lemna* after 3, 5 and 7 days of exposure.
Measurements were conducted with AAS (see material and methods); measurements are based on 4 independent replicates. Error bars give the standard deviation, asterisks indicate significant differences (t-test, α=0.05) between the concentration at t=3 and the indicated exposure period

3.2.3 Predicting toxicity over time

Haber, Bliss and Ostwald/Dernoscheck

Herbicides

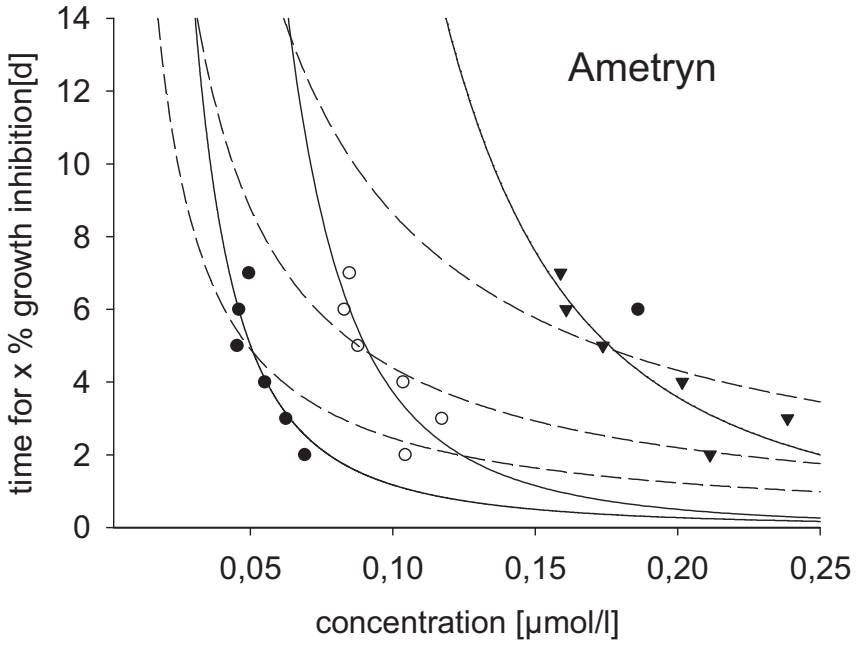


Figure 7: Toxicity of Ametryn over time fitted with the equations of Haber, Bliss and Ostwald/Dernoscheck.
The black circles represent the EC_{25} values, white circles the EC_{50} values and the black triangles the EC_{75} values derived from the concentration-response curves of single substance tests over time from three up to seven days of exposure. The EC values were fitted with either the Haber equation ($c \cdot t = k$) shown here as a dashed line, the Bliss equation ($c \cdot t^{\gamma} = k$) shown as a dotted line or the Ostwald/Dernoscheck equation ($c^{\gamma} \cdot t = k$) shown as a solid line. Generally the curve-progressions of the Bliss and the Ostwald/Dernoscheck fits are identical and thus the curves overlap.

The relation of toxicity and time could be described by the Haber, Bliss and Ostwald/Dernoscheck equation provided that the toxicity increased over time. Within the investigated time scale, the data could be sufficiently fitted especially with the equations of Bliss and/or Ostwald/Dernoscheck shown for Ametryn for an example (Figure 7). Alachlor was the only exception, as the Haber equation provided a better fit for the EC_{25} and EC_{50} data (annex Figure 59A). The curve progression of the Ostwald/Dernoscheck and Bliss fit were generally identical. The data of Aclonifen and Paraquat could be well characterized by the simple Haber equation as well as the Bliss and Ostwald/Dernoscheck equations (annex Figure 58A and Figure 61A). If the toxicity was small or toxicity was nearly constant over time, as it was the case for Ametryn and to some extent Alachlor, the Haber curve correlated badly with the data (Figure 7 and Figure 59A). In the extreme, the Haber curve crossed the data points grossly describing the toxicity-time relation. Depending on the accuracy of the fits, toxicity

was underestimated for short exposure durations and overestimated at long exposure durations regardless whether the Haber fit or the Bliss and Ostwald/Dernoscheck fit was taken.

Though the experiments were conducted under so called ideal conditions of continuous exposure problems fitting the data emerged. This was the case if toxicity decreased over time, as it was the case for Prometon or did not show any clear time trend as it was the case for Diuron (annex Figure 60A and Figure 62A).

The power term γ shown in Table 8 as used in the Bliss equation describes the weight of the factor time i.e. γ describes to which extent time determines toxicity. The γ values for Ametryn were all smaller than one. This indicates that the toxicity of Ametryn was more concentration-dependent than time-dependent. The γ values ranged around 0,4. This was also the case for Paraquat. However, the γ values increased with increasing effect level from 0,6 to 1,03. The toxicity of Alachlor was clearly time-dependent. The γ values were well above one and increased with increasing effect level from 3,74 to 5,64. For Aclonifen the γ values were slightly above one with values of 1,28 for EC_{25} and 1,17 for EC_{50}.

	γ (EC_{25})	γ (EC_{50})	γ (EC_{75})
Ametryn	0,48	0,35	0,38
Aclonifen	1,28	1,17	
Alachlor	3,74	1,66	5,64
Paraquat	0,60	0,74	1,03

Table 8 The power terms γ of the herbicides investigated:
The power term γ derives from the Bliss equation $k= c*t^{\gamma}$ which was used to describe the relationship between toxicity and time (see attachment Figure 58A-62A and Figure 7). The power term γ describes to which extent time determines toxicity.

Metals

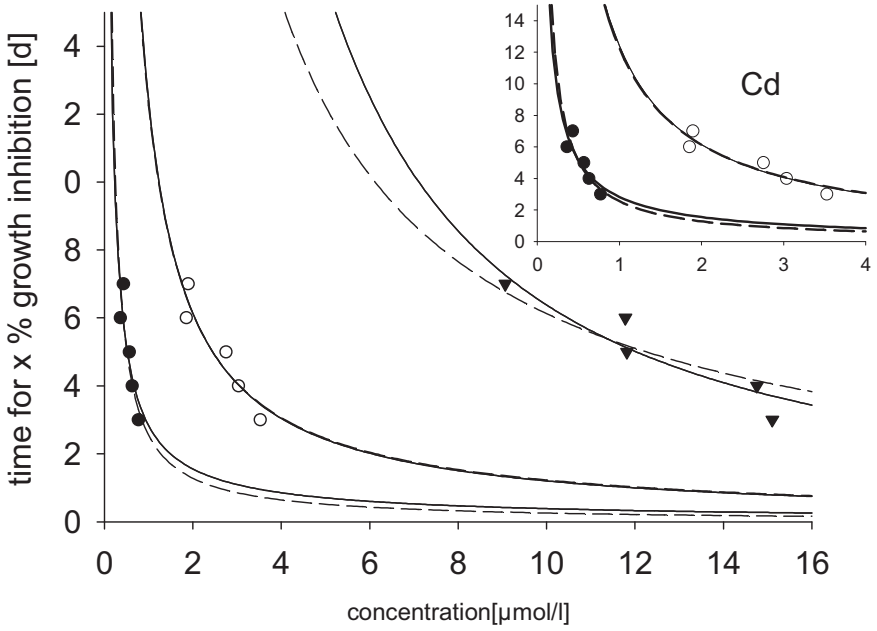


Figure 8: Toxicity of cadmium over time fitted with the equations of Haber, Bliss and Ostwald/Dernoscheck.
The black circles represent the EC_{25} values, white circles the EC_{50} values and the black triangles the EC_{75} values derived from the concentration-response curves of single substance tests over time from three up to seven days of exposure. The EC values were fitted with either the Haber equation ($c \cdot t = k$) shown here as a dashed line, the Bliss equation ($c \cdot t^{\gamma} = k$) shown as a dotted line or the Ostwald/Dernoscheck equation ($c^{\gamma} \cdot t = k$) shown as a solid line. Generally the curve-progressions of the Bliss and the Ostwald/Dernoscheck fits are identical and thus the curves overlap.

Similarly to the herbicides, the relation of toxicity and time could be described by the Haber, Bliss and Ostwald/Dernoscheck equation provided that the toxicity increased over time. Within the investigated time scale, the data could be sufficiently fitted especially with the equations of Bliss and/or Ostwald/Dernoscheck as shown for cadmium as an example (Figure 8). The data of cadmium and the EC_{25} data of nickel could be well characterized by the simple Haber equation as well as the Bliss and Ostwald/Dernoscheck equations (Figure 8 and Figure 64A). If the increase of toxicity was small or toxicity was nearly constant over time, as it was the case copper and to some extent for nickel, or if as in the case of zinc toxicity strongly increased, the Haber curve correlated badly with the data (annex Figure 63A; Figure 64A and Figure 65A). In the extreme, the Haber curve crossed the data points grossly describing the toxicity-time relation. With the exception of zinc, toxicity was underestimated for short exposure durations and overestimated at long exposure durations. In the case of zinc, toxicity was overestimated for short exposure durations but and underestimated for long exposure durations.

The power term γ shown in Table 9 as used in the Bliss equation describes the weight of the factor time i.e. γ describes to which extent time determines toxicity. According to the increasing toxicity over time, zinc had the highest γ values, which were approximately four for the EC_{25} and EC_{50} values. The γ (EC_{75}) value was 1,27. For nickel and cadmium the γ values decreased with increasing effect level from 0,84 to 0,25 for nickel and from 1,16 to 0,76 for cadmium. In the case of copper the γ values were similar for all three regarded effect levels and the values were 0,84, 0,31 and 0,34.

	γ (EC_{25})	γ (EC_{50})	γ (EC_{75})
Zn	3,96	4,51	1,27
Cu	0,38	0,31	0,34
Ni	0,84	0,45	0,25
Cd	1,16	1	0,76

Table 9: The power terms γ of the metals investigated:
The power term γ derives from the Bliss equation $k= c*t^{\gamma}$ which was used to describe the relationship between toxicity and time (see attachment Figure 63A-65A and Figure 8). The power term γ describes to which extent time determines toxicity.

3.3 Variable exposure to single substances

3.3.1 Observed recovery potential after a single pulse exposure

Herbicides

For the recovery experiments, the plants were exposed to concentrations causing an approximate effect of 50 percent after three days. The effect of Aclonifen, Alachlor and Paraquat after a three-day pulse was not reversible whereas Diuron exposed showed fast recovery (Figure 9).There was a slight recovery of the plants exposed to Aclonifen whereas in the case of Paraquat and Alachlor the growth rate even decreased. If however the plants were exposed to a pulse with a lower concentration of Alachlor a recovery was recordable (Figure 10). In the case of Diuron, the recovery pattern after a pulse with different concentrations was similar though the recovery at low concentrations seems to be slower but the standard deviation is high. Plants exposed to Ametryn showed a very good recovery. Even if the plants were exposed to extraordinary high concentrations of Ametryn (more than 15 times the EC_{50} value) recovery could be recorded. Similar results have been observed for the recovery after Prometon exposure.

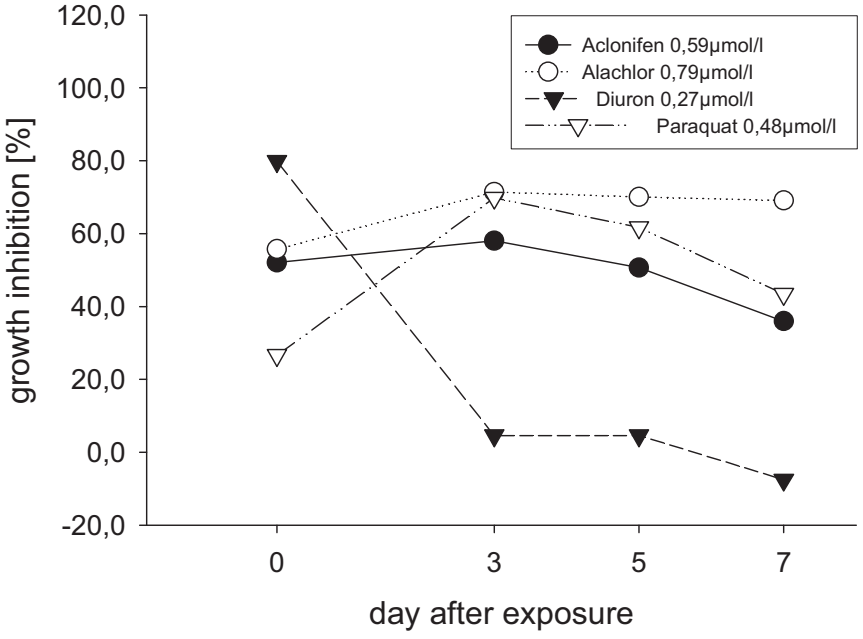


Figure 9: Recovery of *Lemna minor* after three-day exposure to the indicated herbicides.
The plants were exposed for 3 days to a concentration that inhibited the reproduction by approximately 50%. Afterwards they were transferred to fresh, uncontaminated medium and the growth was recorded for another seven days

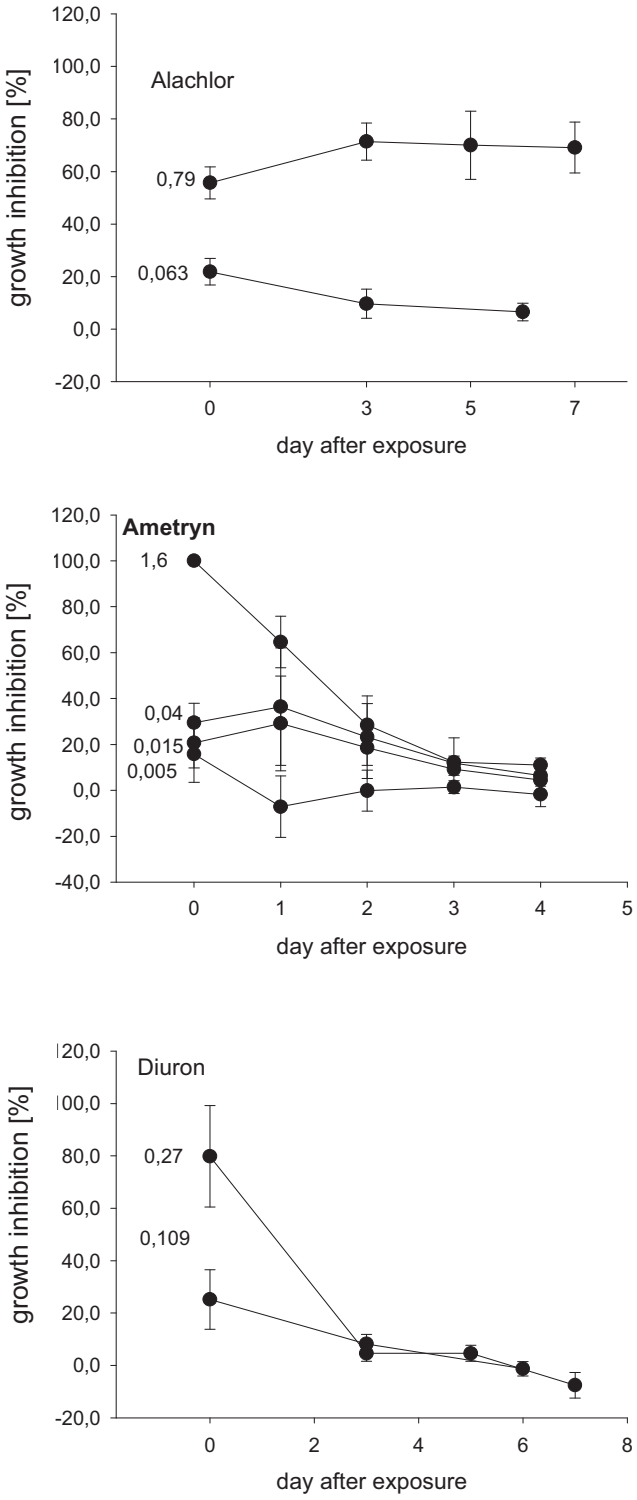


Figure 10: Recovery of *Lemna minor* after an exposure to Alachlor, Ametryn or Diuron
Over 3 days at the indicated concentrations. Experiments were conducted with four (Alachlor and Diuron) and three (Ametryn) replicates. Concentrations are given in µmol/l.

Metals*

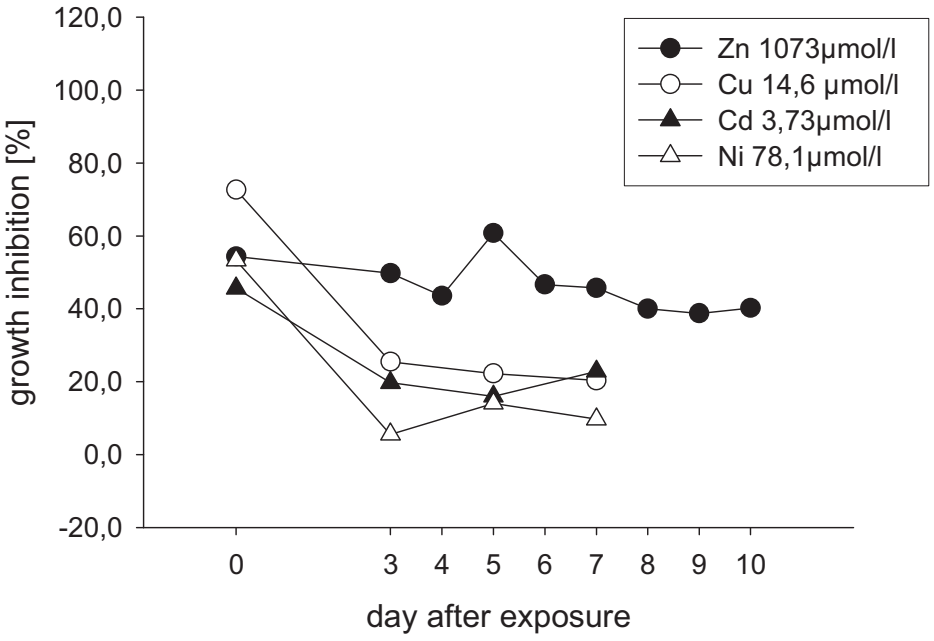


Figure 11: Recovery of *Lemna minor* after three-day exposure to the indicated heavy metals. The plants were exposed for 3 days to a concentration that inhibited the reproduction by approximately 50%. Afterwards they were transferred to fresh, uncontaminated medium and the growth was recorded for another seven days.

Metal-specific recovery patterns, after the plants had been exposed to EC_{50}-concentrations for 3 days, were observed (Figure 11). Even ten days after transferring *Lemna minor* into uncontaminated medium the zinc pre-exposed plants still showed a severely reduced growth rate. Copper and cadmium pre-exposed *Lemna* showed growth rates close to control level after 3 days recovery, while nickel pre-exposed *Lemnas* actually reached control levels. If pulses with higher concentrations causing an approximate effect of 75% growth inhibition were applied, no recovery was observed from copper and zinc whereas the plants showed full recovery after pulses with low concentrations. In the case of copper, the plants showed even better growth than the controls (Figure 12).

* Parts of this chapter and all figures except figure 12 have already been published elsewhere; see: Drost W, Matzke M, Backhaus T (2007): Heavy metal toxicity to *Lemna* minor: studies on the time-dependence of growth inhibition and the recovery after exposure. Chemosphere 67(1): 36-43

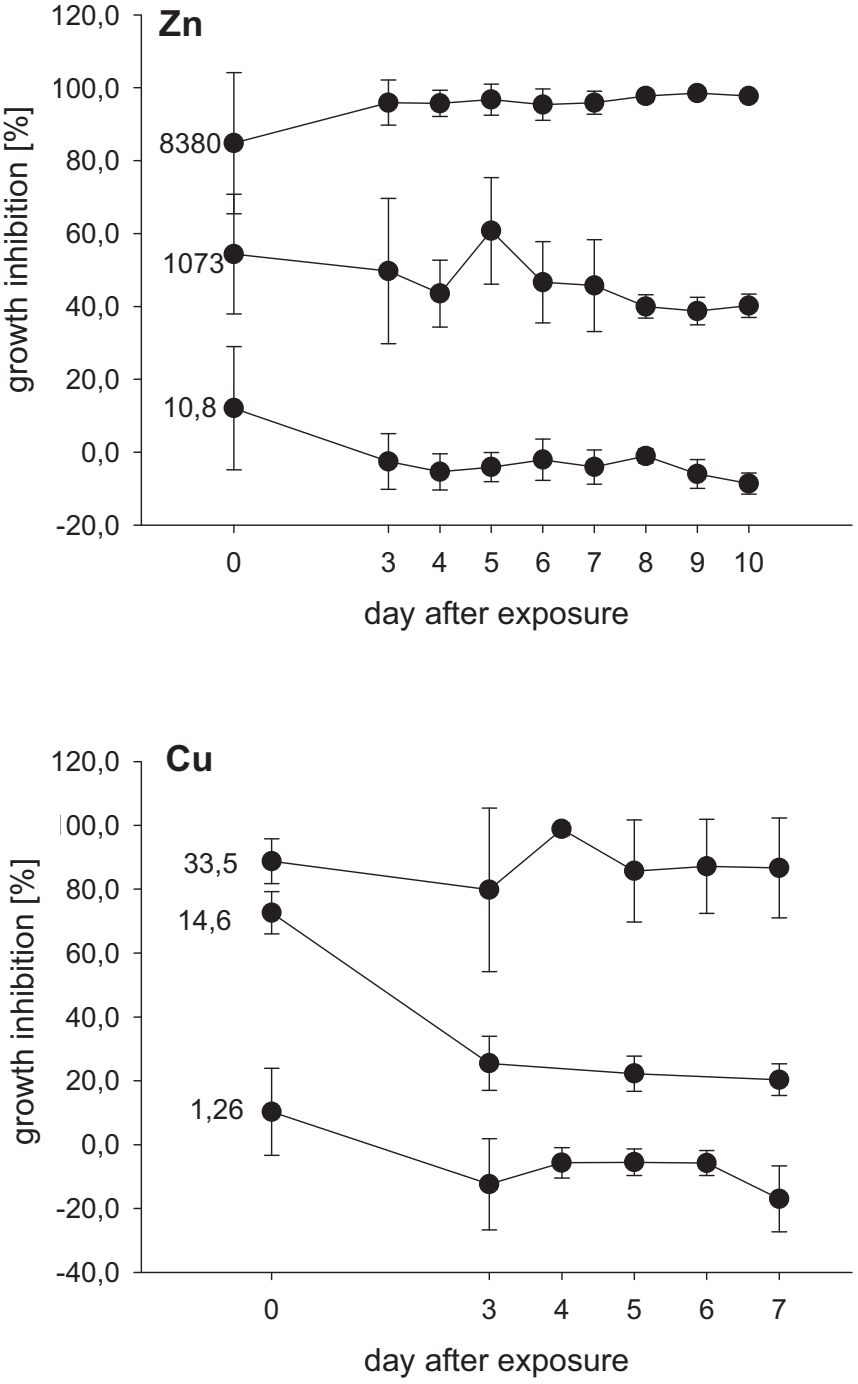


Figure 12: Recovery of *Lemna minor* after an exposure to Cu or Zn over 3 days at the indicated concentrations. Experiments were conducted with three replicates. Concentrations are given in µmol/l

Internal concentration after pulsed exposure

As expected, the internal concentrations of all metals decreased during the recovery phase (Figure 13). Nevertheless, metal concentrations were still significantly above control levels even 7 days after ending the exposure. In comparison to the other metals, nickel-concentrations decreased rapidly. Three days after the exposure phase, concentrations were already down to only 20% of the internal concentration that were found during the exposure phase. In contrast, the zinc concentration was still at 92%.

In principle, two processes might be responsible for the decrease in metal concentrations: (a) active or passive excretion of the metal from the plant and (b) the "dilution" of the internal metal concentration due to an increase in total biomass (growth). In order to distinguish between these two processes, internal metal concentrations were re-calculated on a per-sample basis (Figure 14). A significant decrease was only detectable for copper (factor 2) and nickel (factor 3), while the total amount of zinc and cadmium in the plants remained constant. The decrease in cadmium and zinc concentrations on a dry weight basis seems to be completely due to the increase in biomass. The total amount of these two metals in the biomass of each sample did not change over a recovery period of 7 days.

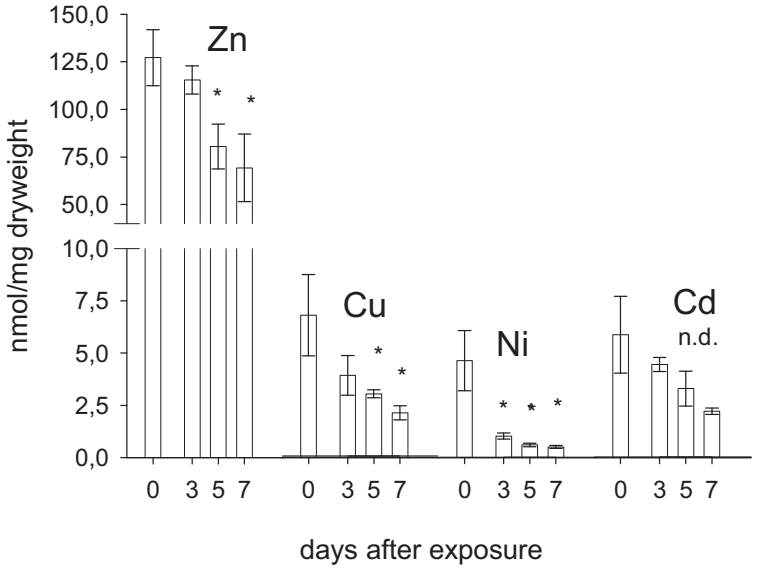


Figure 13: Development of the internal metal concentrations during the recovery of *Lemna* from metal pre-exposure referred to dry weight.
Given are the internal metal concentrations in nmol/mg dry weight. Asterisks give statistical significant differences (t-test, α=0.05) between the concentration at t=3 and the indicated recovery period. For the cadmium experiment, no significances were determined as one of the samples from t=3 were lost during the sample preparation.

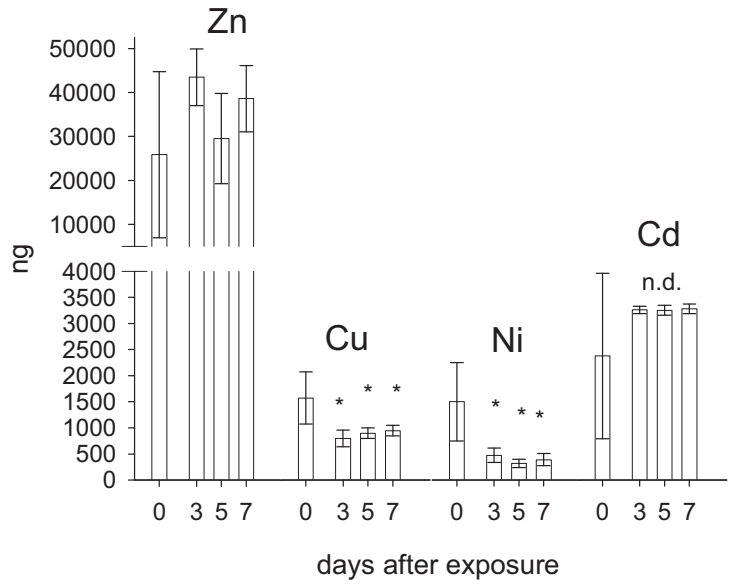


Figure 14: Development of the internal metal concentrations during the recovery of *Lemna minor* from metal pre-exposure referred to the total biomass per sample.
Given are the internal metal concentrations in the total biomass per sample. Asterisks give statistical significant differences (t-test, α=0.95) between the concentration at t=3 and the indicated recovery period. For the cadmium experiment, no significances were determined as one of the samples from t=3 was lost during the sample preparation

3.3.2 Observed toxicity of fluctuating exposure to Alachlor, copper or Diuron

All plants exposed to a three day pulse of Alachlor, Diuron or copper showed recovery, if no subsequent second pulse of exposure followed (Figure 15). The plants showed a growth inhibition of 20 to 25%. Especially in the case of a Diuron exposure, the plants showed a fast recovery, whereas the recovery after a copper pulse was delayed. Alachlor exposed plants recovered fast up to day three but the growth inhibition then remained unchanged. At day six only plants that had been exposed to Diuron had fully recovered. Plants that had been exposed to Alachlor or copper still showed a growth inhibition of about 10% six days after the exposure.

The plants were repeatedly exposed to the same substances Alachlor, copper and Diuron. Shown in Figure 16, Figure 17 and Figure 18 are the observed growth inhibitions referred to untreated controls (white circles) and pre-treated control (white triangles). If the growth inhibition was calculated on the basis of untreated controls, data points are indicated as black circles. If calculated on the basis of pre-treated controls, data points are indicated as grey triangles. In order to gain insight into whether plants became more or less sensitive if pre-treated, the data points are plotted with the single substance concentration response curve achieved from standard exposure conditions. The solid line gives the fit to the data, inner dashed lines the approximate 95% confidence belt of the mean, outer dotted lines the 95% confidence belt of the population.

Due to an increasing toxicity of Alachlor over time, the impact of the pre-treatment became especially apparent at day six. A pre-treatment with Alachlor produced highly sensitive plants in particular. The pre-exposure to Alachlor had special impact on the sensitivity in the low dose area (Figure 16). If growth inhibition was calculated on the basis of the Alachlor pre-treated control plants, the growth inhibition was slightly lower but nevertheless higher than compared to the sensitivity of plants which had not been pre-treated.

As the recovery from a copper pulse occurred slowly, growth inhibition referred to copper pre-treated control plants led to a concentration response relationship to copper which was less sensitive than the dose relationship of untreated plants at day three in the low dose area (Figure 17). All pre-treated plants which were exposed to concentrations causing an approximate growth inhibition up to 20% were less sensitive than those plants which had not been pre-exposed. This is indicated by the data points (grey triangle) which lie under the concentration response curve. If growth inhibition was calculated on the basis of controls which had not been pre-exposed to copper, data points were within the confidence belt and

the prediction belt but were shifted above the concentration response curve. Regarding day six, there was no difference whether growth inhibition was calculated on the basis of pre-treated or untreated control plants. Pre-treated plants showed an increased sensitivity towards copper, but the data points were nevertheless within the prediction and confidence belt.

Plants pre-exposed to Diuron showed an increased sensitivity to Diuron at day three, but this effect diminished over time as the pre-treatment showed no effect at day six (Figure 18). Due to the good recovery of a Diuron pulse, pre-treated controls were in respect to their growth rate similar to the untreated controls and thus growth inhibition of Diuron treated plants were similar if referred to pre-treated or untreated control plants.

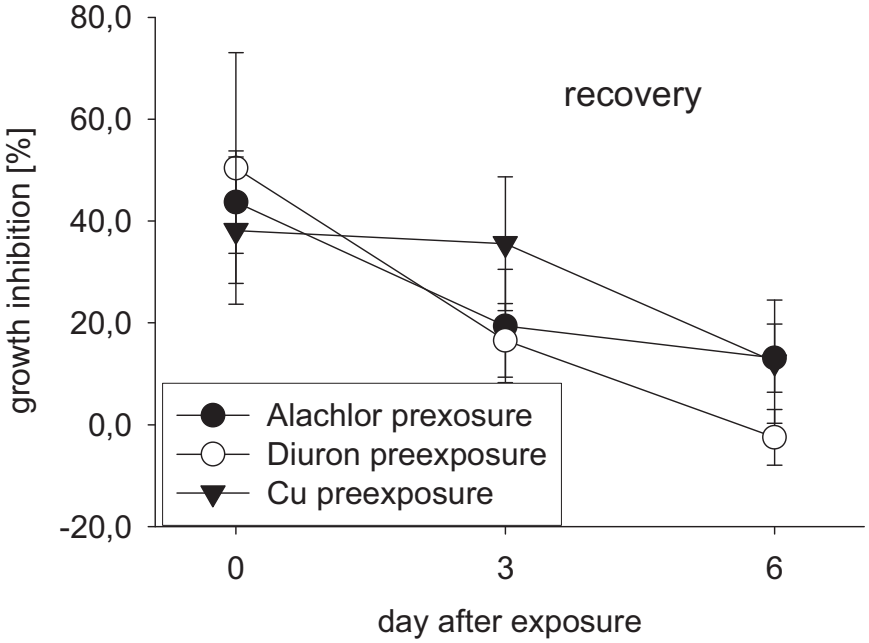


Figure 15: Recorded recovery after a three day pulse to Alachlor (0,063 µmol/l), Diuron (0,109 µmol/l) or copper (0,673 µmol/l) in concentrations causing a growth inhibition of approximately 30%. Experiments were conducted with four replicates.

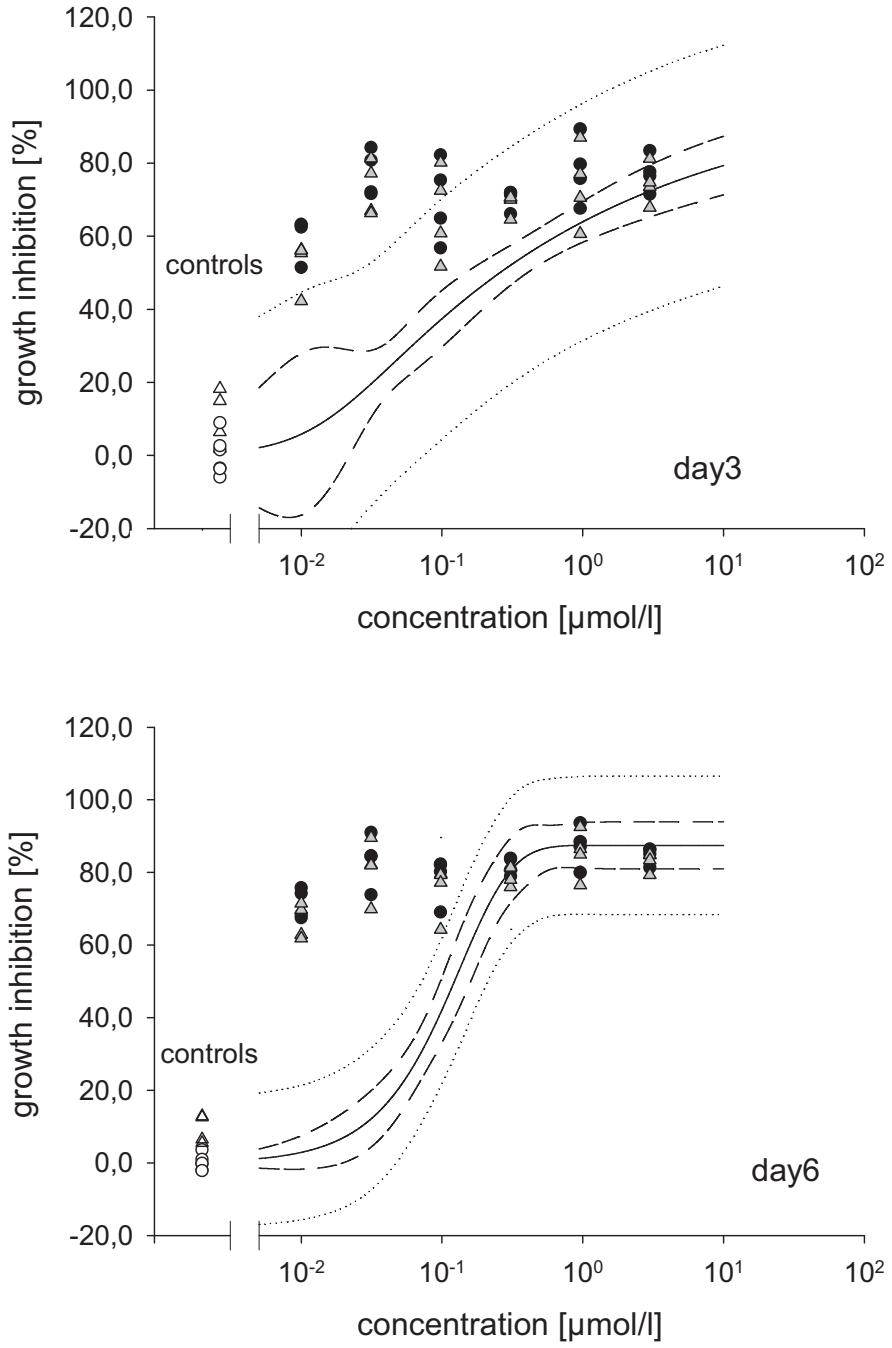


Figure 16: Observed concentration response relationship of Alachlor after a pre-treatment with Alachlor: The plants were pre-treated with Alachlor for three days. The concentration of 0,063 µmol/l caused approximately 30% growth inhibition. Subsequently the plants were transferred to various concentrations of Alachlor and were exposed up to six days. For comparison the data gained are plotted with the single substance dose-response curve of Alachlor (solid line) achieved with plants not pre-treated. Additionally, the 95% confidence belt (dashed line) of the single substance response curve and the 95% prediction belt (dotted line) of the single substance response data are shown. Black circles represent the data of growth inhibition referred to untreated control plants (white circles). Grey triangles represent the data of the growth inhibition referred to pre-treated control plants (white triangles).

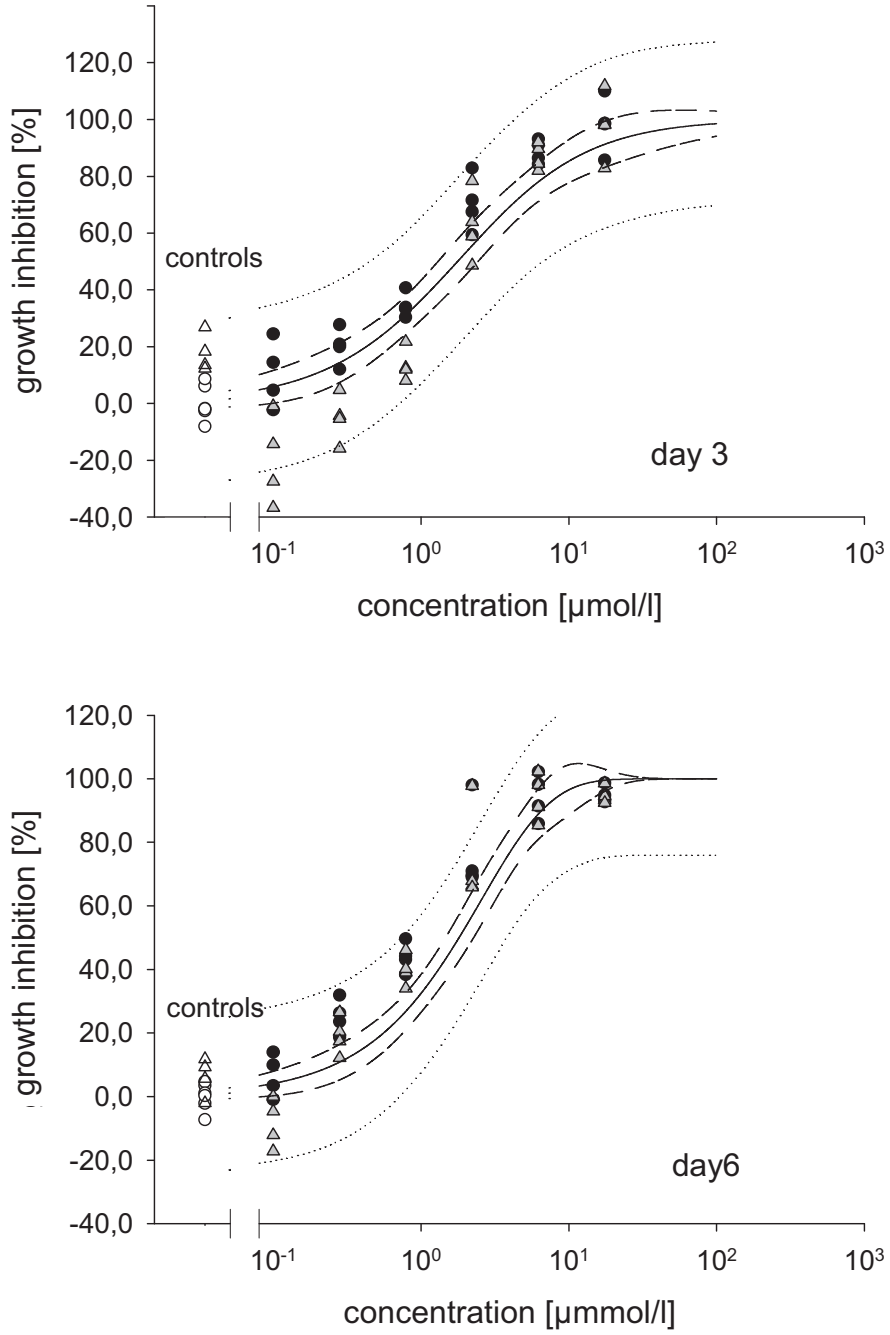


Figure 17: Observed concentration response relationship of copper after a pre-treatment with copper. The plants were pre-treated with copper for three days. The concentration of 0,673 µmol/l caused approximately 30% growth inhibition. Subsequently the plants were transferred to various concentrations of Alachlor and were exposed up to six days. For comparison the data gained are plotted with the single substance dose-response curve of copper (solid line) achieved with plants not pre-treated. Additionally, the 95% confidence belt (dashed line) of the single substance response curve and the 95% prediction belt (dotted line) of the single substance response data are shown. Black circles represent the data of growth inhibition referred to untreated control plants (white circles). Grey triangles represent the data of the growth inhibition referred to pre-treated control plants (white triangles).

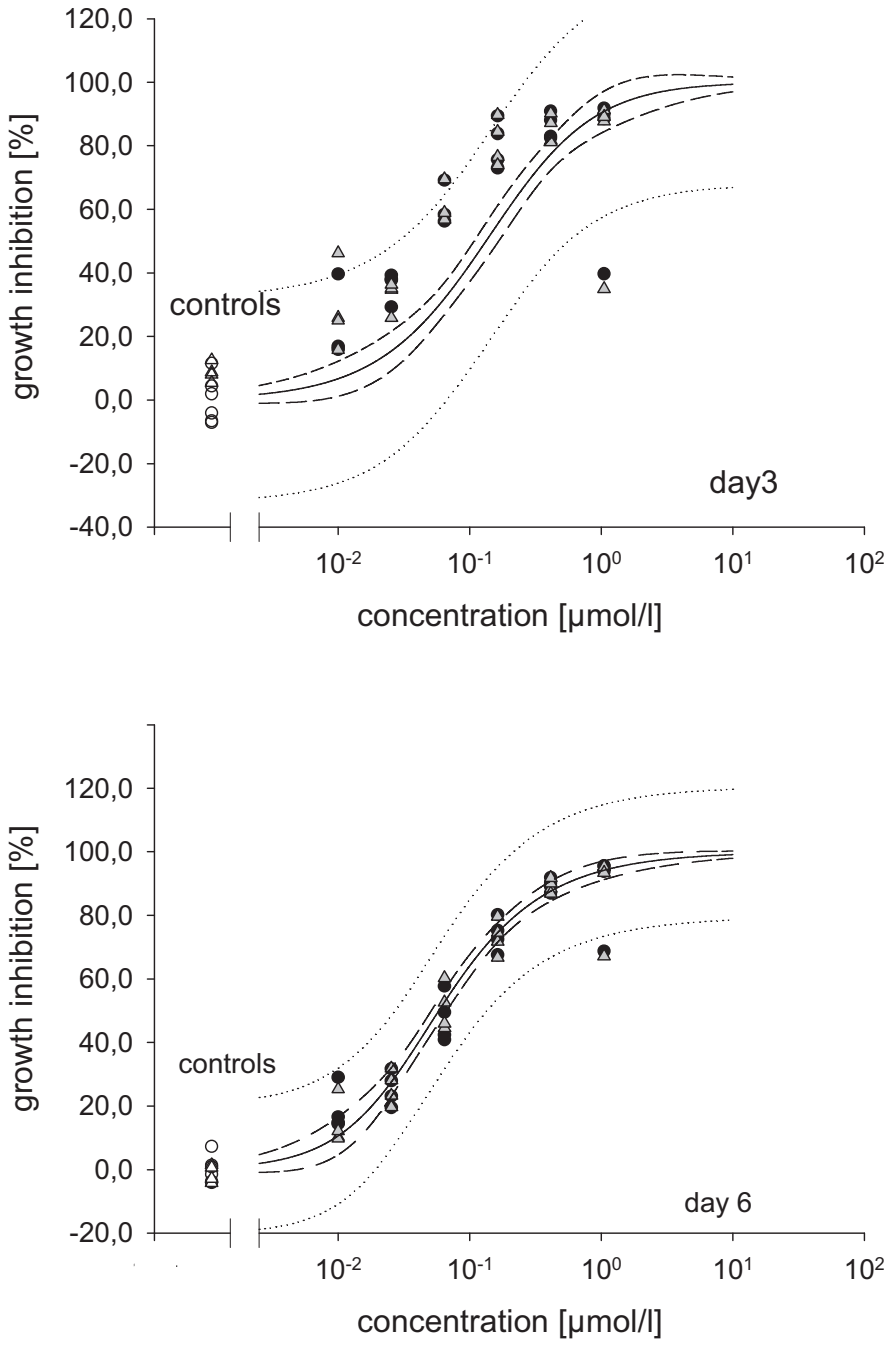


Figure 18: Observed concentration response relationship of Diuron after a pre-treatment with Diuron. The plants were pre-treated with Diuron for three days. The concentration of 0,109 µmol/l caused approximately 30% growth inhibition. Subsequently the plants were transferred to various concentrations of Alachlor and were exposed up to six days. For comparison the data gained are plotted with the single substance dose-response curve of Diuron (solid line) achieved with plants not pre-treated. Additionally, the 95% confidence belt (dashed line) of the single substance response curve and the 95% prediction belt (dotted line) of the single substance response data are shown. Black circles represent the data of growth inhibition referred to untreated control plants (white circles). Grey triangles represent the data of the growth inhibition referred to pre-treated control plants (white triangles).

3.4 Mixture Toxicity under simple and complex exposure conditions

3.4.1 Observed and predicted toxicity of mixtures of Alachlor, copper and Diuron of constant composition

	γ (EC_{25})	γ (EC_{50})	γ (EC_{75})	Observed EC_{50}	Predicted EC_{50}	
					Concentration Addition	Independent Action
	Alachlor and Cu					
3 days	0,6358	1,9746	2,1281	0,2666	1,013	0,623
6 days				0,0956	0,966	1,021
	Alachlor and Diuron					
3 days	0,5191	1,7926	2,0762	0,0437	0,191	0,376
6 days				0,0204	0,104	0,104
	Cu and Diuron					
3 days	0,9343	0,8144	0,8564	0,6854	1,013	0,623
6 days				0,3398	0,966	1,021
	Alachlor. Cu and Diuron					
3 days				0,8058	0,717	0,442
6 days				0,8660	0,718	0,479

Table 10: Observed and predicted EC_{50} values of the two and three-component mixture of Alachlor, Cu and Diuron.
The mixture ratio is referred to the EC_{50} values of the individual components. Values are given in µmol/l. The power term γ derives from the Bliss equation k= c*t γ which was used to describe the relationship between toxicity and time. The power term γ describes to which extent time determines toxicity.

The simultaneous mixture experiments were conducted exposing *Lemna minor* to two or all three substances in the ratio of their EC_{50} values. Referring to the single substances, the power terms of Alachlor were all well above one for all three effect levels regarded, whereas the power terms of copper were well below one. This indicates that the toxicity is time-determined in the case of Alachlor and more concentration-determined in the case of copper. The toxicity of Diuron decreased over time and hence could not be fitted. The results of the

power terms for the description of the mixture toxicity over time indicate that the time dependency of the overall toxicity is determined by its mixture components and their single substance toxicity-time relationship (Table 10). For mixtures consisting of two substances and Alachlor as one substance the power terms were above one indicating that the mixture toxicity was more time-determined than concentration-determined. In agreement with the observations made for the single substance toxicity, mixture toxicity was more concentration-determined than time-determined in the case of the copper-Diuron-mixture. For the mixture with all three substances the observed data could not be fitted with the Haber equations and its deviation as the toxicity decreased over time.

The simultaneous mixture experiments were conducted exposing *Lemna minor* to two or all three substances in the ratio of their EC_{50} values. The predictions of the mixture toxicity whether based on CA or IA were similar but the IA predictions were always shifted to lower concentrations than the CA predictions.

For the Alachlor and copper mixture the toxicity was underestimated in the low dose area (Figure 19). Effects of 20 to 40% growth inhibition were observed at day three whereas no effect was predicted for this concentration. The prediction by means of the IA concept produced a better fit to the obtained data. This was also the case at day six. The observed toxicity as well as the predicted toxicity increased over time. The observed EC_{50} value changed from 0,731 to 0,186 µmol/l which is a factor of four. The predicted EC_{50} values changed with a factor of 2,7 and 2,6 (Table 10). CA generally underestimated the toxicity over the whole range of concentration whereas IA gave a good prognosis for the high dose area.

The same was observed if Alachlor was combined with Diuron (Figure 20). The toxicity of the low dose area was underestimated for day three whereas the toxicity was generally underestimated for day six. The prediction by means of IA was generally better. The predicted EC_{50} remained unchanged over time for the Alachlor and Diuron mixture whereas the observed EC_{50} value at day three decreased to about one third at day six (Table 10). Thus the underestimation of the mixture toxicity increased over time.

The predictions fitted well for the copper Diuron mixture and the Alachlor, copper and Diuron mixture (Figure 21 and Figure 22). A slight increase of toxicity of the copper and Diuron mixture was observed which was also predicted by CA and IA. The observed EC_{50} value changed from 0,714 to 0,406 µmol/l which is a factor of 1,7 (Table 10). IA slightly over

assessed the EC_{50} value at day three and under estimated theEC_{50} value at day six, whereas CA underestimated the toxicity for both time points. The observed mixture toxicity with all three components as well as the estimated EC_{50} values did not change over time.

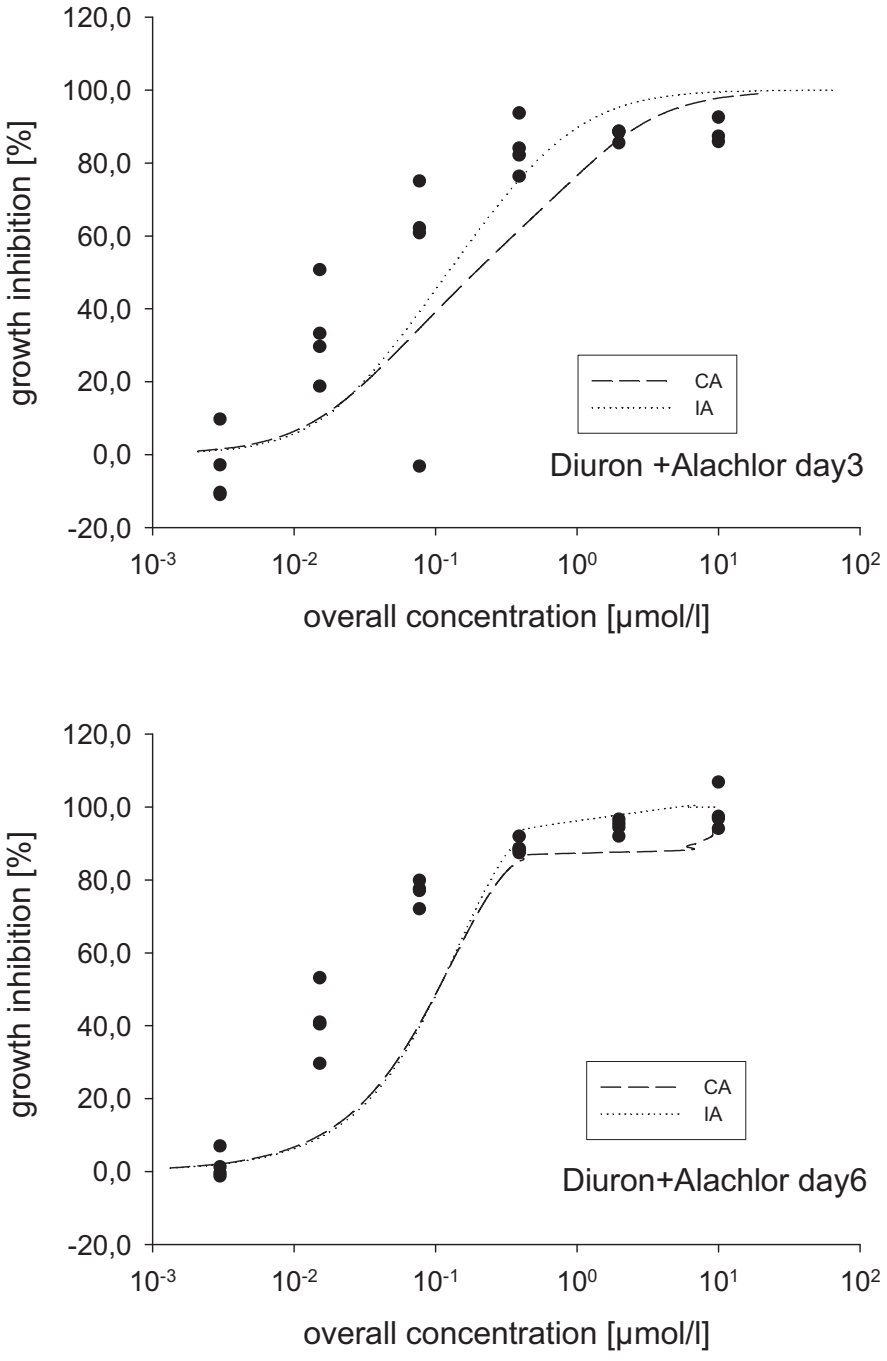


Figure 19: Observed and predicted toxicity of a binary mixture with Alachlor and Diuron.

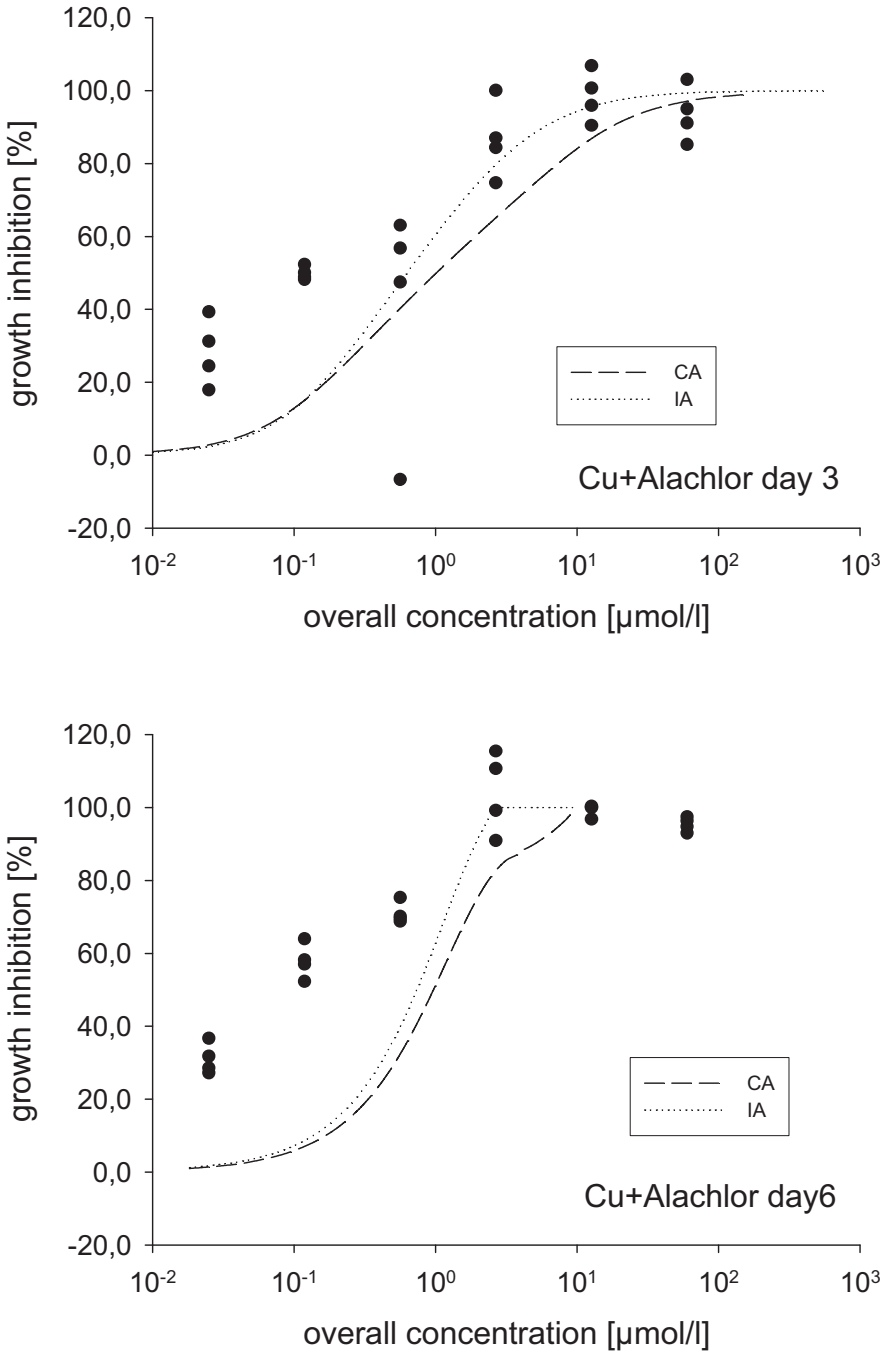


Figure 20: Observed and predicted toxicity of a binary mixture with Alachlor and copper.

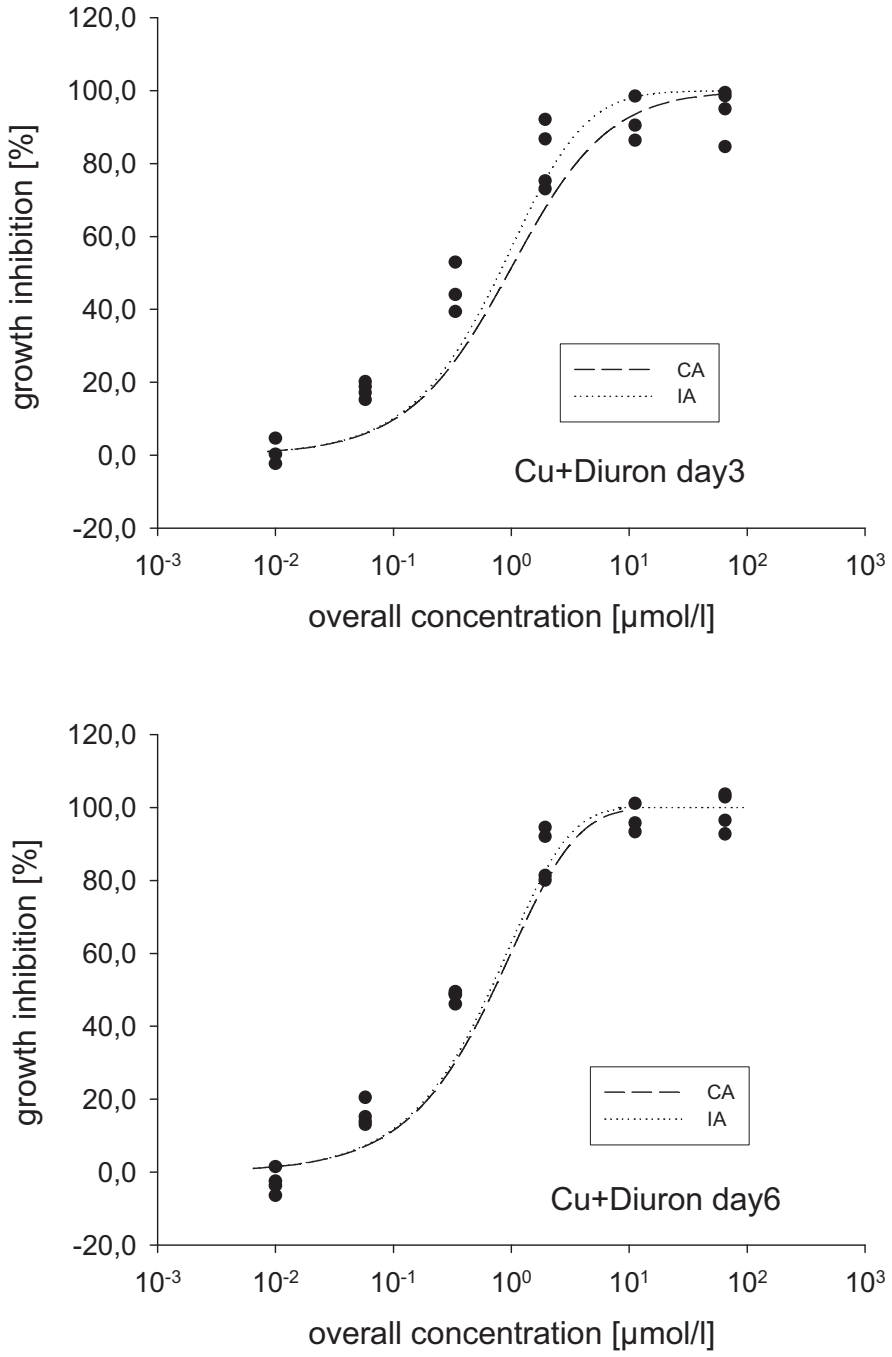


Figure 21: Observed and predicted toxicity of a binary mixture with copper and Diuron.

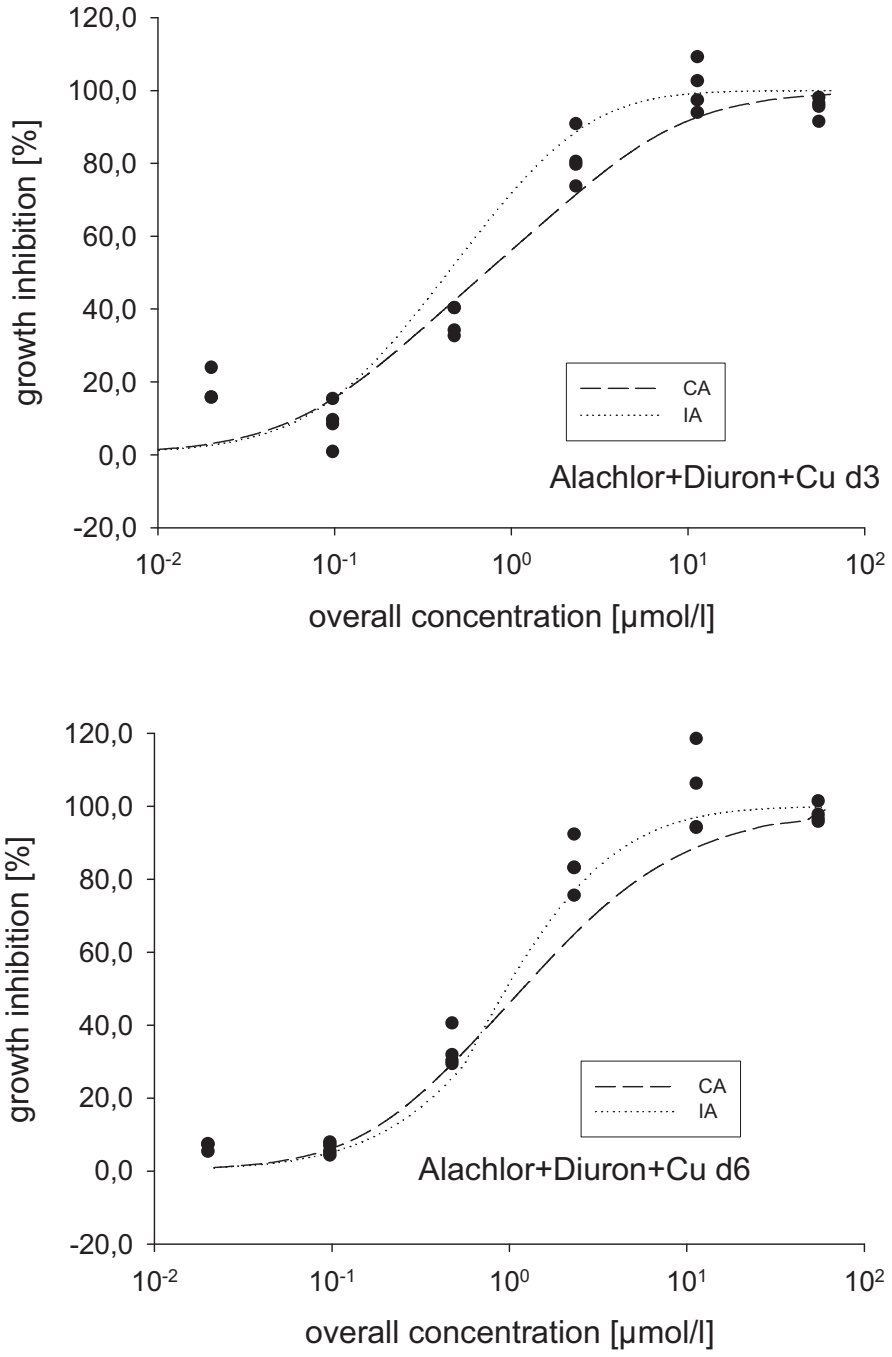


Figure 22: Observed and predicted toxicity of the mixture with Alachlor, copper and Diuron

3.4.2 Observed combination effects of Alachlor, Copper and Diuron in fluctuating compositions

Shown in the following Figure 23 to Figure 28 are the observed growth inhibitions referred to untreated controls (white circles) and pre-treated control (white triangles). If growth inhibition was calculated on the basis of untreated controls, data points are indicated as black circles. If calculated on the basis of pre-treated controls data points are indicated as grey triangles. In order to gain insight into whether plants became more or less sensitive if pre-treated, the data points are plotted with the single substance concentration response curve achieved from standard exposure conditions. The solid line gives the fit to the data, inner dashed lines the approximate 95% confidence belt of the mean, outer dotted lines the 95% confidence belt of the population.

Influence of Diuron or copper pre-position on the concentration response relationship of Alachlor

Referred to untreated controls there was an increase in the sensitivity of the plants to Alachlor if pre-exposed to Diuron or copper for three days in concentrations causing approximately 30% growth inhibition (Figure 23 and Figure 24). Due to the pre-exposure to EC30 the recorded growth inhibition was 30% at minimum for Alachlor concentrations having no or little impact on the growth of *Lemna minor* without pre-treatment. The data points of the pre-exposure experiments at day three were within the prediction belt obtained from experiments with untreated plants but not in the confidence belt of the Alachlor fit describing the concentration response relationship for Alachlor (Figure 23 and Figure 24). The pre-exposure to copper or Diuron had special impact on the sensitivity in the low dose area. Though *Lemna minor* recovered quickly from a three day pulse of Diuron (Figure 10 and Figure 15) a pre-treatment nevertheless raised the sensitivity of the plants. The Diuron pre-exposure even caused an increase in sensitivity over time. The Alachlor concentration causing a growth inhibition of 10% to untreated plants caused a growth inhibition of approximately 30% at day three and a growth inhibition of 50% at day six. In the case of copper there was also an increase of sensitivity over time. The Alachlor concentration causing a growth inhibition of 20% to untreated plants caused a growth inhibition of approximately 40% at day three and a growth inhibition between 60% and 70% at day six.

If growth inhibition was referred to the growth of plants which also had been exposed to a three day pulse of Diuron or copper, than the recorded growth inhibition was slightly reduced. Yet, due to a fast recovery of the plants after a Diuron pulse (Figure 10 and Figure 15) the

difference between untreated and pre-treated control plants and thus the difference between the calculated growth inhibitions based on pre-treated or untreated control plants was small. However, in the case of a copper pre-exposure and as the recovery after a copper pulse the did not occur as fast, the growth inhibition referred to pre-treated control plants resulted in a dose relationship of Alachlor nearly equal to the concentration response relationship of plants with no pre-treatment at day three (Figure 23). Due to an ongoing recovery of the pre-treated control plants the differences of the variable calculated growth-inhibition diminished at day six.

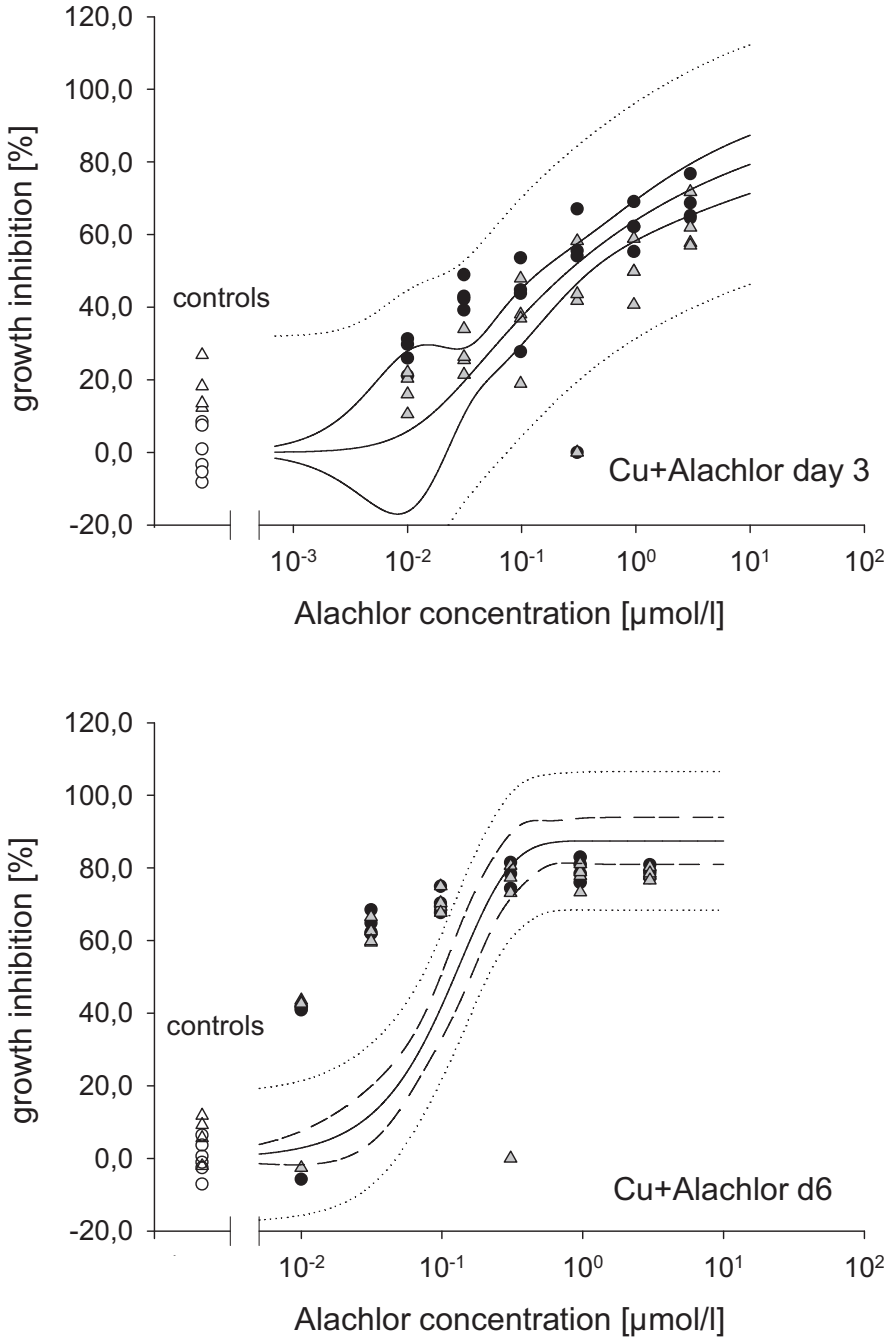


Figure 23: Observed concentration response relationship of Alachlor after a pre-treatment with copper: The plants were pre-treated with copper for three days. The concentration of 0,673 µmol/l caused approximately 30% growth inhibition. Subsequently the plants were transferred to various concentrations of Alachlor and were exposed up to six days. For comparison the data gained are plotted with the single substance dose-response curve of Alachlor (solid line) achieved with plants not pre-treated. Additionally, the 95% confidence belt (dashed line) of the single substance response curve and the 95% prediction belt (dotted line) of the single substance response data are shown. Black circles represent the data of growth inhibition referred to untreated control plants (white circles). Grey triangles represent the data of the growth inhibition referred to pre-treated control plants (white triangles).

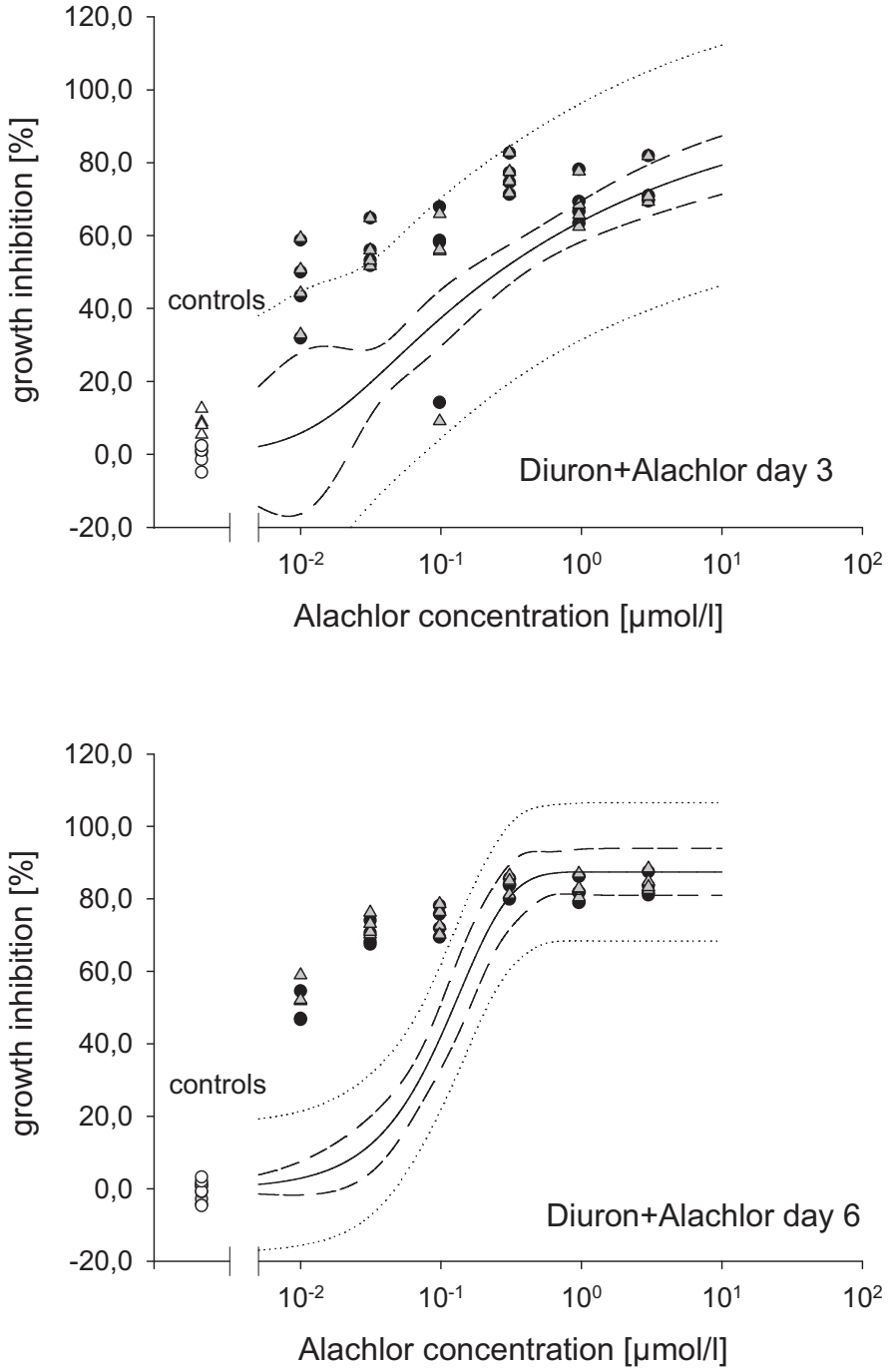


Figure 24: Observed concentration response relationship of Alachlor after a pre-treatment with Diuron. The plants were pre-treated with Diuron for three days. The concentration of 0,109 µmol/l caused approximately 30% growth inhibition. Subsequently the plants were transferred to various concentrations of Alachlor and were exposed up to six days. For comparison the data gained are plotted with the single substance dose-response curve of Alachlor (solid line) achieved with plants not pre-treated. Additionally, the 95% confidence belt (dashed line) of the single substance response curve and the 95% prediction belt (dotted line) of the single substance response data are shown. Black circles represent the data of growth inhibition referred to untreated control plants (white circles). Grey triangles represent the data of the growth inhibition referred to pre-treated control plants (white triangles).

Influence of Alachlor or Diuron pre-exposure on the concentration response relationship of Copper

A pre-exposure with Alachlor or Diuron did not result in such an increase of sensitivity towards copper as was noted for the combination of a copper or Diuron pre-treatment and a subsequent exposure to Alachlor (Figure 25 and Figure 26). There was no recorded minimum growth inhibition of 30%, though plants pre-treated with Diuron did show a slightly increased sensitivity to copper in the low dose area. This was however not so pronounced and the increased sensitivity could only be seen at day three. No changes of sensitivity were recordable for plants exposed to higher copper concentrations at day three and the sensitivity and thus the concentration response relationship at day six remained unchanged. Hence a pre-exposure to Diuron had only small impact if any on the sensitivity to copper. Alachlor pre-treated plants even showed a slight decrease of sensitivity towards copper. This effect was especially pronounced in the high effect region. Compared to the single substance concentration response curve of copper without pre-treatment, the data points gained were all below the concentration response curve but within the prediction belt. The copper concentration causing a growth inhibition of 50% to untreated plants caused a growth inhibition of approximately 10% to 30% at day three and a growth inhibition of approximately 30% at day six.

Due to fast recovery after a pulsed exposure to Diuron, the difference between untreated and pre-treated controls was small and thus the growth inhibition referred to pre-treated control plants showed only little difference to the growth inhibition referred to untreated control plants. Due to the slower recovery from a single Alachlor pulse compared to the rapid recovery from a single Diuron pulse, growth inhibition referred to pre-treated control plants were less pronounced than growth inhibition referred to untreated control plants. This became especially apparent at day six.

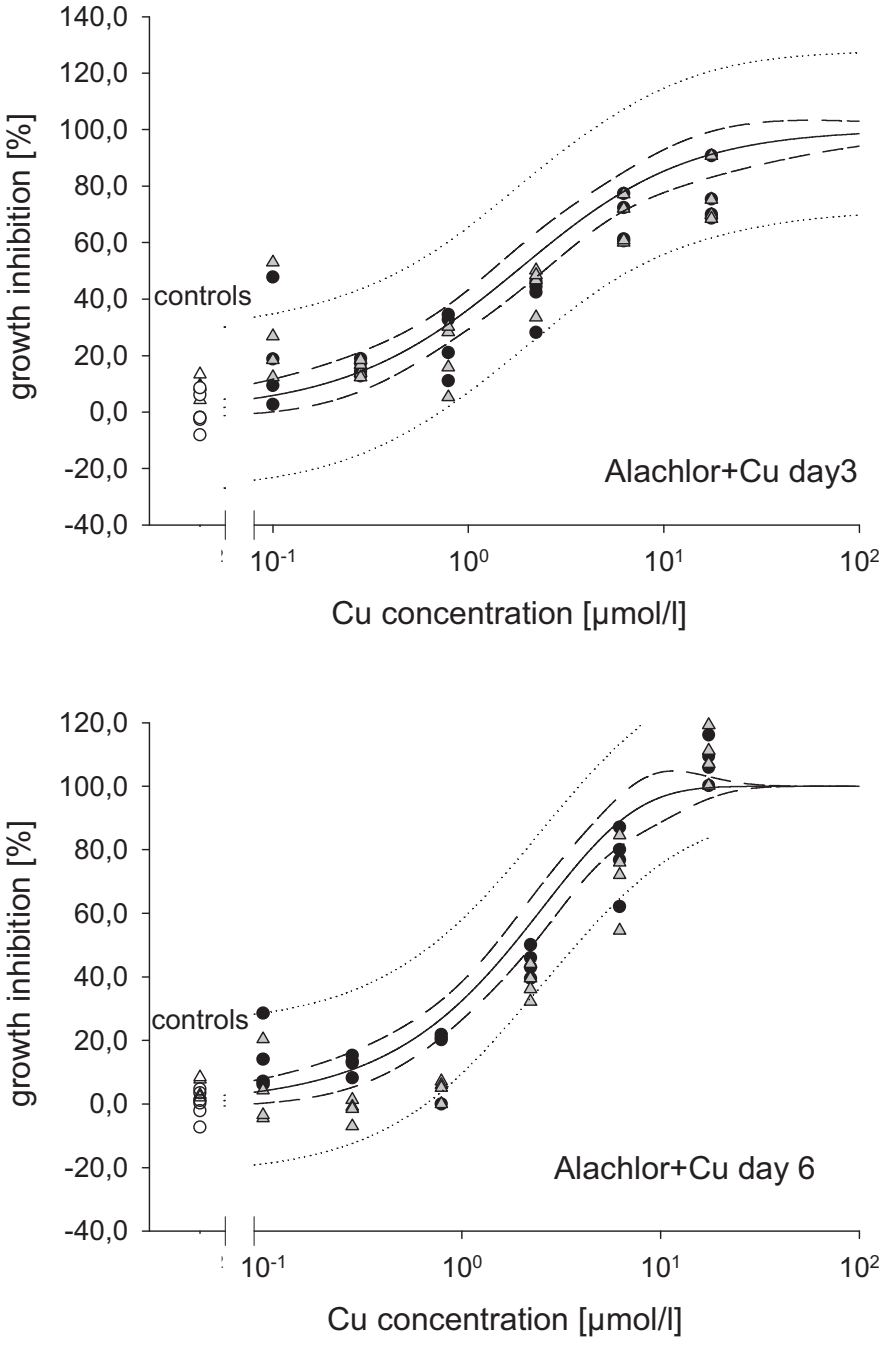


Figure 25: Observed concentration response relationship of copper after a pre-treatment with Alachlor. The plants were pre-treated with Alachlor for three days. The concentration of 0,063 µmol/l caused approximately 30% growth inhibition. Subsequently the plants were transferred to various concentrations of copper and were exposed up to six days. For comparison the data gained are plotted with the single substance dose-response curve of copper (solid line) achieved with plants not pre-treated. Additionally, the 95% confidence belt (dashed line) of the single substance response curve and the 95% prediction belt (dotted line) of the single substance response data are shown. Black circles represent the data of growth inhibition referred to untreated control plants (white circles). Grey triangles represent the data of the growth inhibition referred to pre-treated control plants (white triangles).

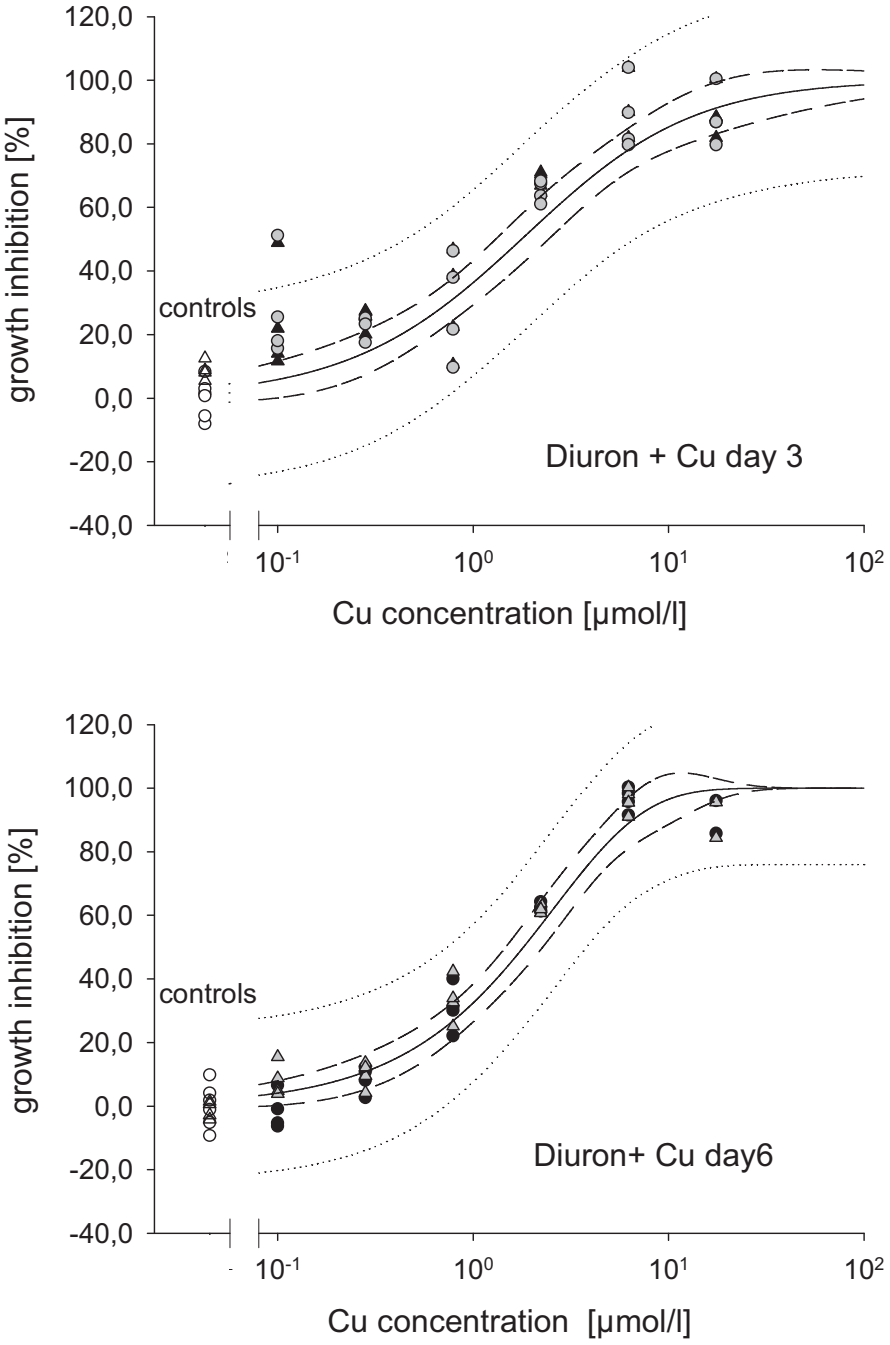


Figure 26: Observed concentration response relationship of copper after a pre-treatment with Diuron. The plants were pre-treated with Diuron for three days. The concentration of 0,109 µmol/l caused approximately 30% growth inhibition. Subsequently the plants were transferred to various concentrations of copper and were exposed up to six days. For comparison the data gained are plotted with the single substance dose-response curve of copper (solid line) achieved with plants not pre-treated. Additionally, the 95% confidence belt (dashed line) of the single substance response curve and the 95% prediction belt (dotted line) of the single substance response data are shown. Black circles represent the data of growth inhibition referred to untreated control plants (white circles). Grey triangles represent the data of the growth inhibition referred to pre-treated control plants (white triangles).

Influence of Alachlor or cu pre-exposure on the concentration response relationship of Diuron

The data gained indicate that the sensitivity to Diuron of the plants generally increased if pre-exposed to Alachlor or copper. (Figure 27 and Figure 28). In both cases the increase of sensitivity became especially pronounced in the low dose area. In the case of copper the sensitivity increased over time. The data gained were outside the prediction belt at day six. The concentration causing a growth inhibition of 20% to untreated plants caused a growth inhibition of approximately 70% at day six. At day three the same concentration caused a growth inhibition approximately 30% to 40%. In the case of Alachlor the sensitivity of the plants decreased slightly over time. The Diuron concentration causing a growth inhibition of 20% to untreated plants caused a growth inhibition of approximately 40% to 60% at day three and a growth inhibition of 40% to 50% at day six.

If growth inhibition was referred to the growth of plants which had also been exposed to a three day pulse of copper then the recorded growth inhibition was slightly reduced compared to the growth inhibition referred to untreated control plants due to a slow recovery from a pulse of copper. Nevertheless, the sensitivity to Diuron was still increased in the case of pre-exposure to copper. In case of pre-exposure to Alachlor, the growth inhibition referred to pre-treated control plants was similar to the growth inhibition referred to untreated control plants.

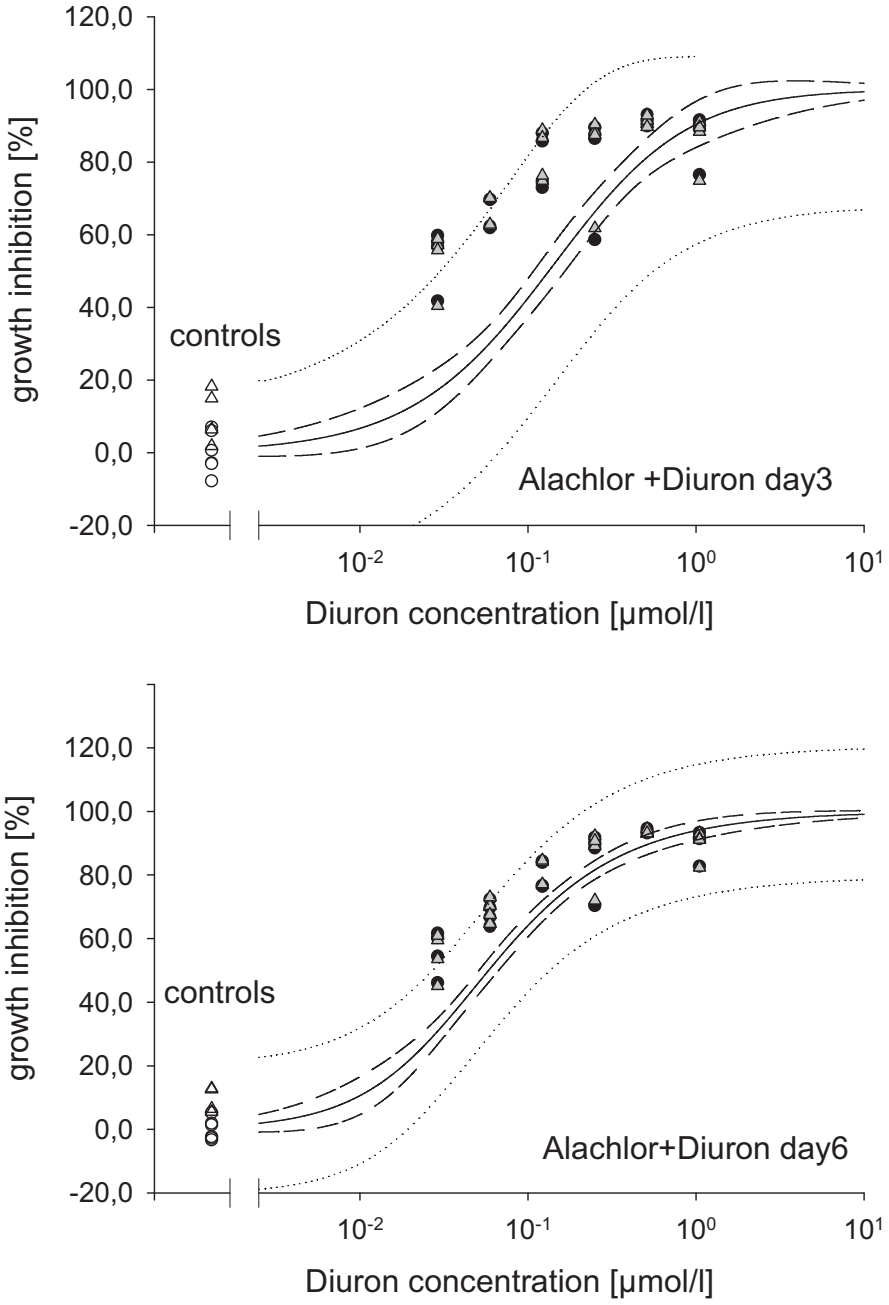


Figure 27: Observed concentration response relationship of Diuron after a pre-treatment with Alachlor. The plants were pre-treated with Alachlor for three days. The concentration of 0,063 µmol/l **caused approximately 30% growth inhibition. Subsequently the plants were transferred to various concentrations of Diuron and were exposed up to six days. For comparison the data gained are plotted with the single substance dose-response curve of Diuron (solid line) achieved with plants not pre-treated. Additionally, the 95% confidence belt (dashed line) of the single substance response curve and the 95% prediction belt (dotted line) of the single substance response data are shown. Black circles represent the data of growth inhibition referred to untreated control plants (white circles). Grey triangles represent the data of the growth inhibition referred to pre-treated control plants (white triangles).**

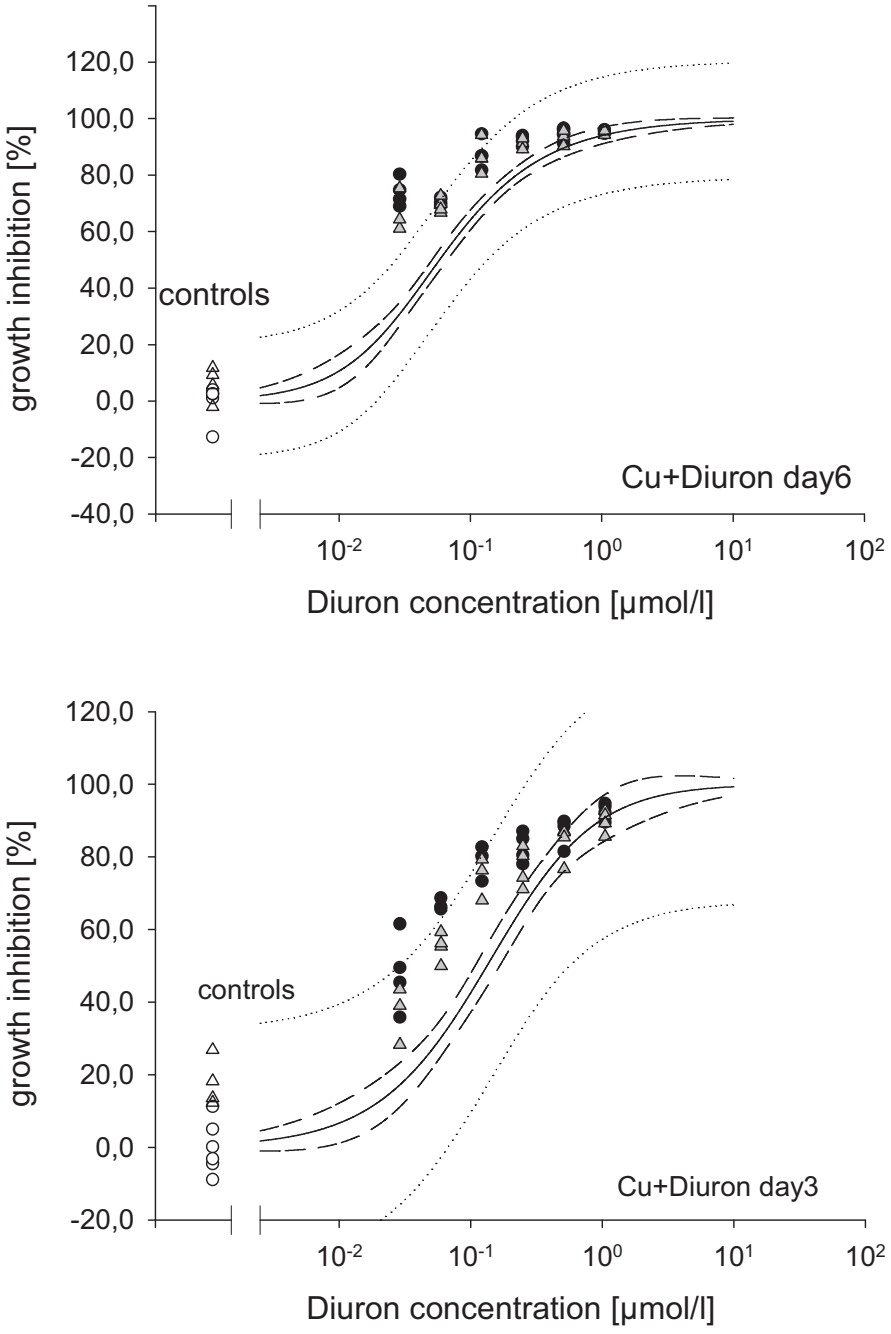


Figure 28: Observed concentration response relationship of Diuron after a pre-treatment with copper. The plants were pre-treated with copper for three days. The concentration of 0,673 µmol/l **caused approximately 30% growth inhibition. Subsequently the plants were transferred to various concentrations of Diuron and were exposed up to six days. For comparison the data gained are plotted with the single substance dose-response curve of Diuron (solid line) achieved with plants not pre-treated. Additionally, the 95% confidence belt (dashed line) of the single substance response curve and the 95% prediction belt (dotted line) of the single substance response data are shown. Black circles represent the data of growth inhibition referred to untreated control plants (white circles). Grey triangles represent the data of the growth inhibition referred to pre-treated control plants (white triangles).**

3.5 Predicted toxicity of mixtures of Alachlor, Copper and Diuron of fluctuating composition

Though the plants were not exposed to mixtures but to single substances sequentially the concepts CA and IA were applied as a mixture may occur in the organism. Due to the experimental design with a pre-exposure to a concentration causing approximately 30% growth inhibition a minimum effect of 30% was predicted. In case of an increasing or decreasing toxicity over time the predictions varied so the predicted minimum became smaller (copper) or larger (Alachlor and Diuron) (Figure 29 to Figure 34). Contrary to the simultaneous exposure to various substances the experiment was not designed on the basis of a prediction but the predictions were made on the basis of the experiments, which means in the case of CA the effects were calculated to the given concentrations iteratively. Due to this way of calculating the effect for the highest concentration could not be predicted for CA. Generally IA predicted higher effects than CA. There were more cases of underestimation of toxicity than overestimation.

For all sequential experiments including Alachlor as the second substance the predicted effects increased from day three to day six. Effects between 50% and 90% were observed but an effect of 30% at day three and an effect of 44% at day six were predicted (Figure 29 and Figure 30). CA gave a good prediction of the toxicity if copper was the substance of pre-exposure, whereas IA overestimated the toxicity. If the plants were pre-exposed to Diuron IA gave a better estimation of the toxicity than CA on day three but the opposite was the case on day six.

The predictions for sequential experiments including copper as the second substance all overestimated the toxicity especially for the lower concentrations (Figure 31 and Figure 32). As an Alachlor as well as a Diuron pre-exposure did not lead to an increased sensitivity to copper or even decreased the sensitivity as was the case for Alachlor, the toxicity was overestimated.

CA and IA provided a good prediction of the toxicity of the Alachlor and subsequent Diuron exposure on *Lemna minor* at day six (Figure 33). The toxicity at day three was underestimated. Effects of approximately 60% growth inhibition were observed where CA and IA assessed a growth inhibition of 40%. The toxicity of the copper and Diuron combination was also underestimated especially at day six (Figure 34). Effects between 70% and 80% growth inhibition were observed but 25% growth inhibition had been predicted for day six.

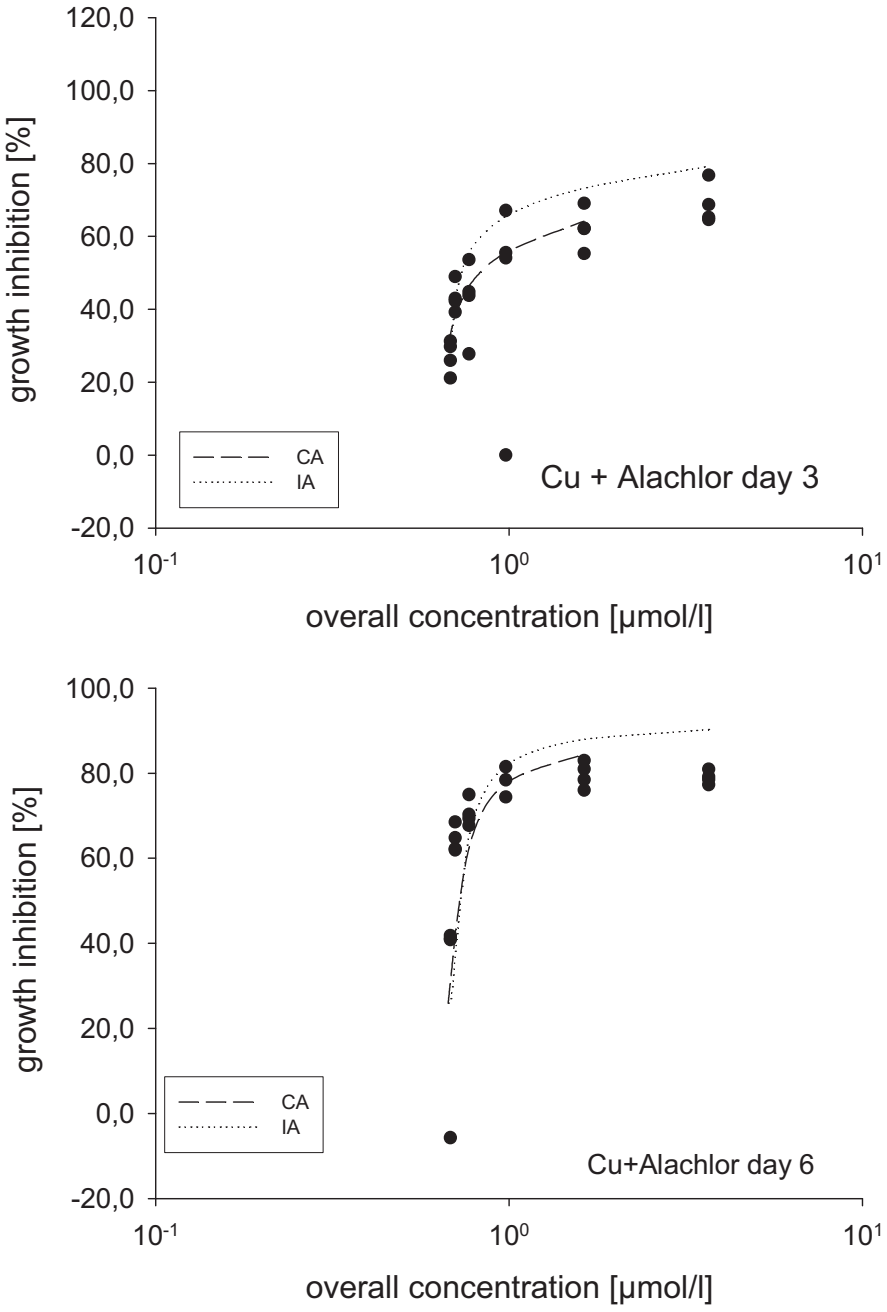


Figure 29: Prediction of the toxicity of two sequential exposure pulses of Cu and Alachlor.
The plants were pre-treated with Cu for three days in a concentration causing approximately 30% growth inhibition (0,673 µmol/l). Subsequently the plants were transferred to a medium with various concentrations of Alachlor and were exposed up to six days. The concentration is the overall concentration of Cu and Alachlor the plants had been exposed to sequentially.

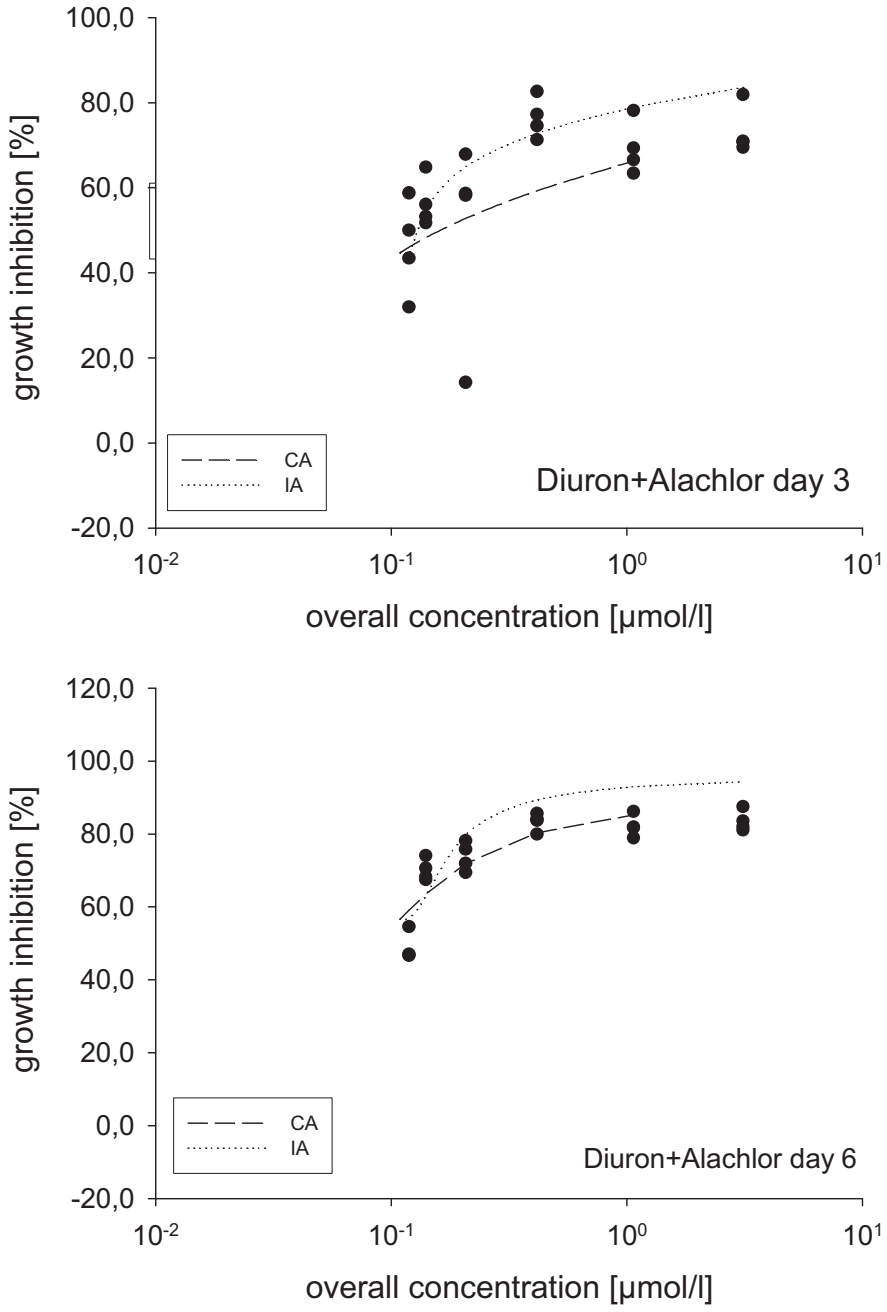


Figure 30: Prediction of the toxicity of two sequential exposure pulses of Diuron and Alachlor.
The plants were pre-treated with Diuron for three days in a concentration causing approximately 30% growth inhibition (0,109 µmol/l). Subsequently the plants were transferred to a medium with various concentrations of Alachlor and were exposed up to six days. The concentration is the overall concentration of Diuron and Alachlor the plants had been exposed to sequentially.

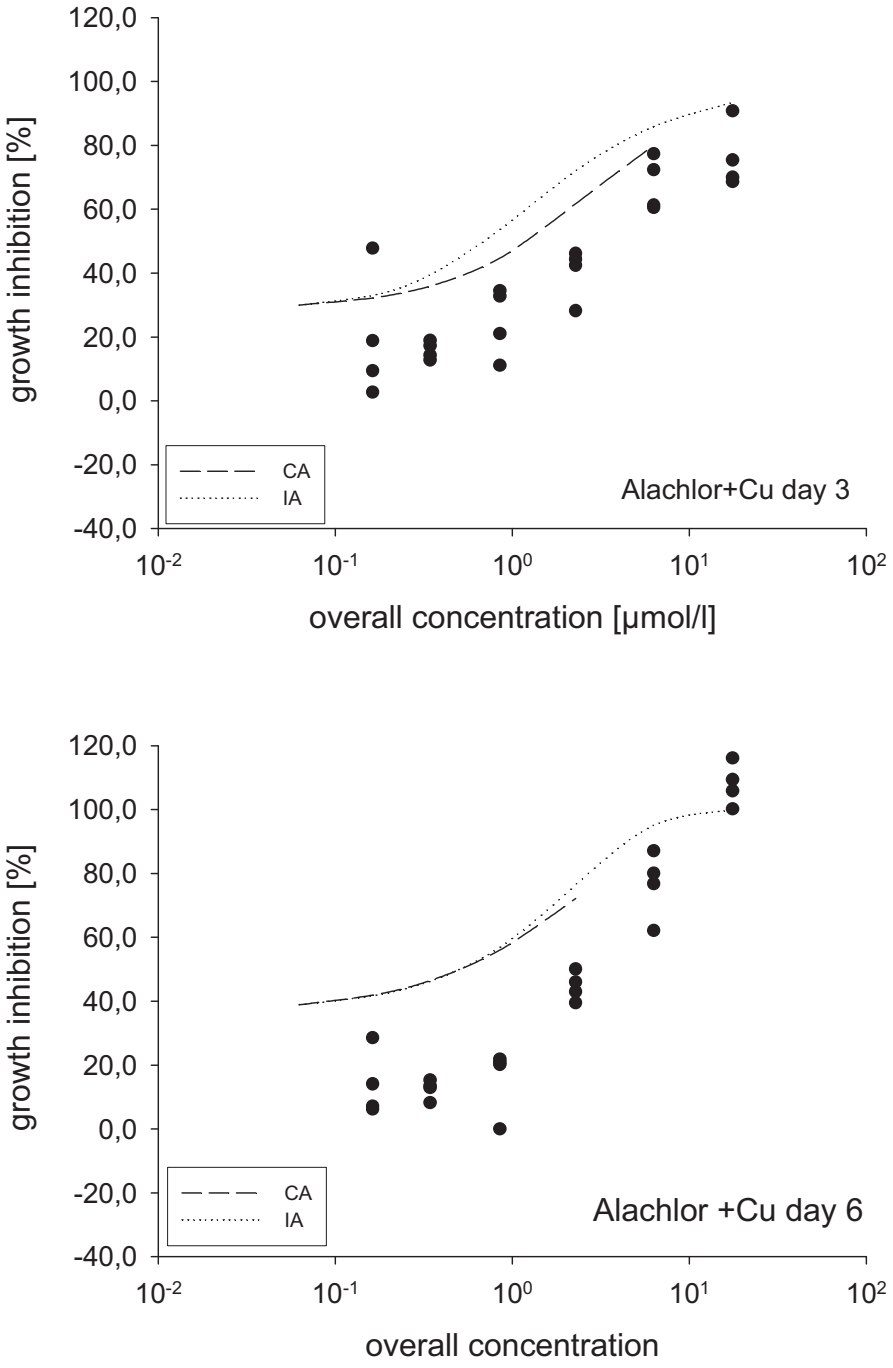


Figure 31: Prediction of the toxicity of two sequential exposure pulses of Alachlor and Cu.
The plants were pre-treated with Alachlor for three days in a concentration causing approximately 30% growth inhibition (0,063 µmol/l). Subsequently the plants were transferred to a medium with various concentrations of Cu and were exposed up to six days. The concentration is the overall concentration of Alachlor and Cu the plants had been exposed to sequentially.

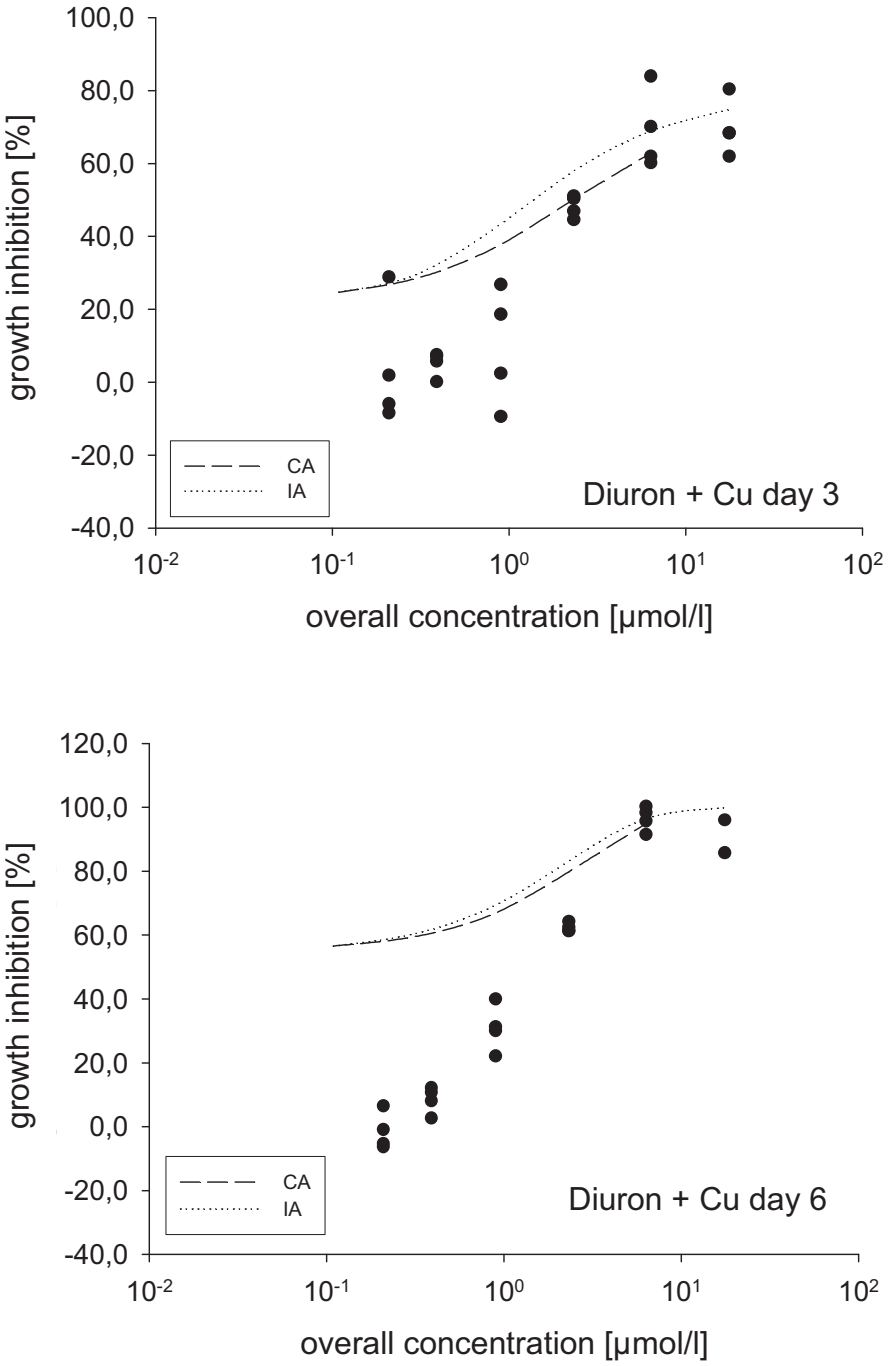


Figure 32: Prediction of the toxicity of two sequential exposure pulses of Diuron and copper.
The plants were pre-treated with Diuron for three days in a concentration causing approximately 30% growth inhibition (0,109 µmol/l). Subsequently the plants were transferred to a medium with various concentrations of Cu and were exposed up to six days. The concentration is the overall concentration of Diuron and copper the plants had been exposed to sequentially.

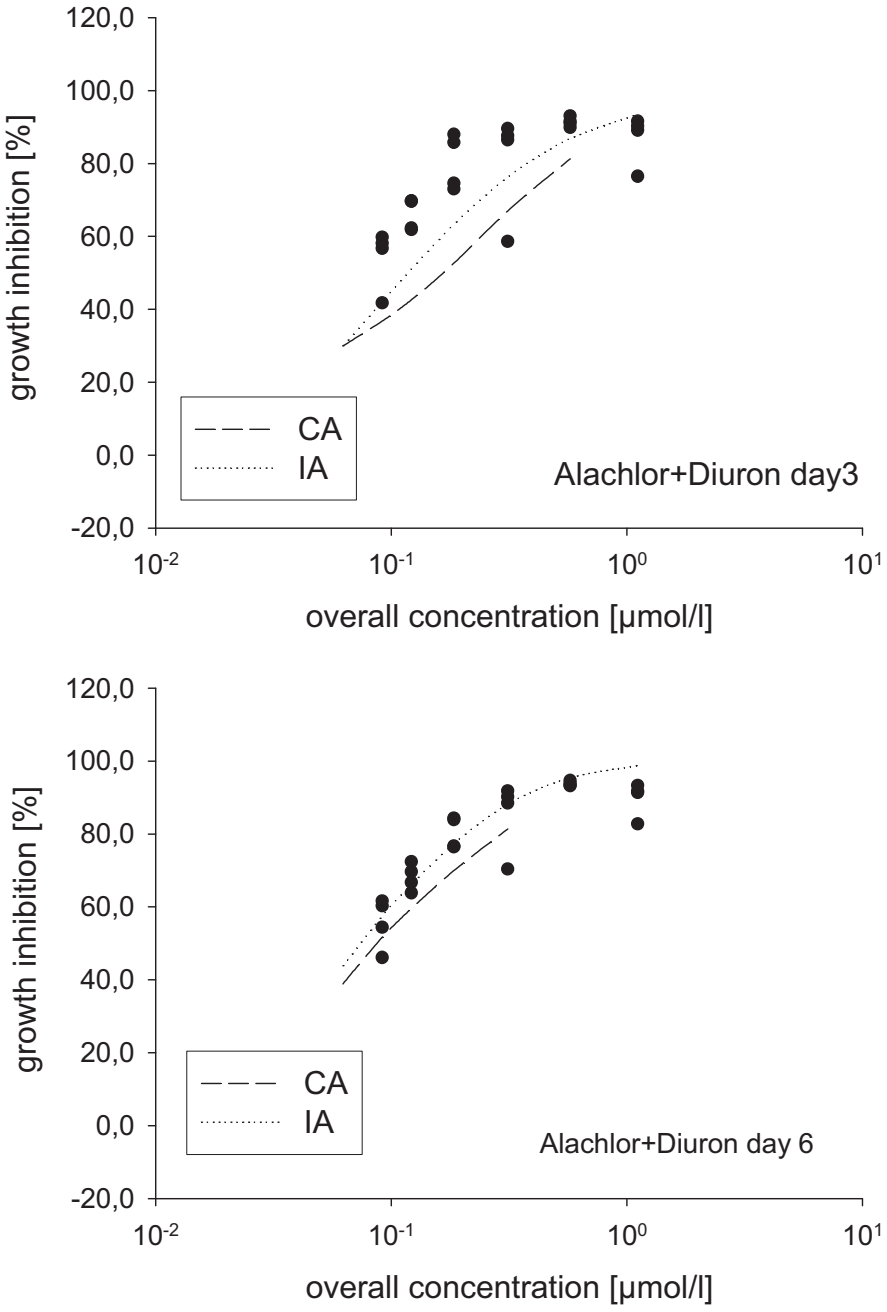


Figure 33: Prediction of the toxicity of two sequential exposure pulses of Alachlor and Diuron.
The plants were pre-treated with Alachlor for three days in a concentration causing approximately 30% growth inhibition (0,063 µmol/l). Subsequently the plants were transferred to a medium with various concentrations of Diuron and were exposed up to six days. The concentration is the overall concentration of Alachlor and Diuron the plants had been exposed to sequentially.

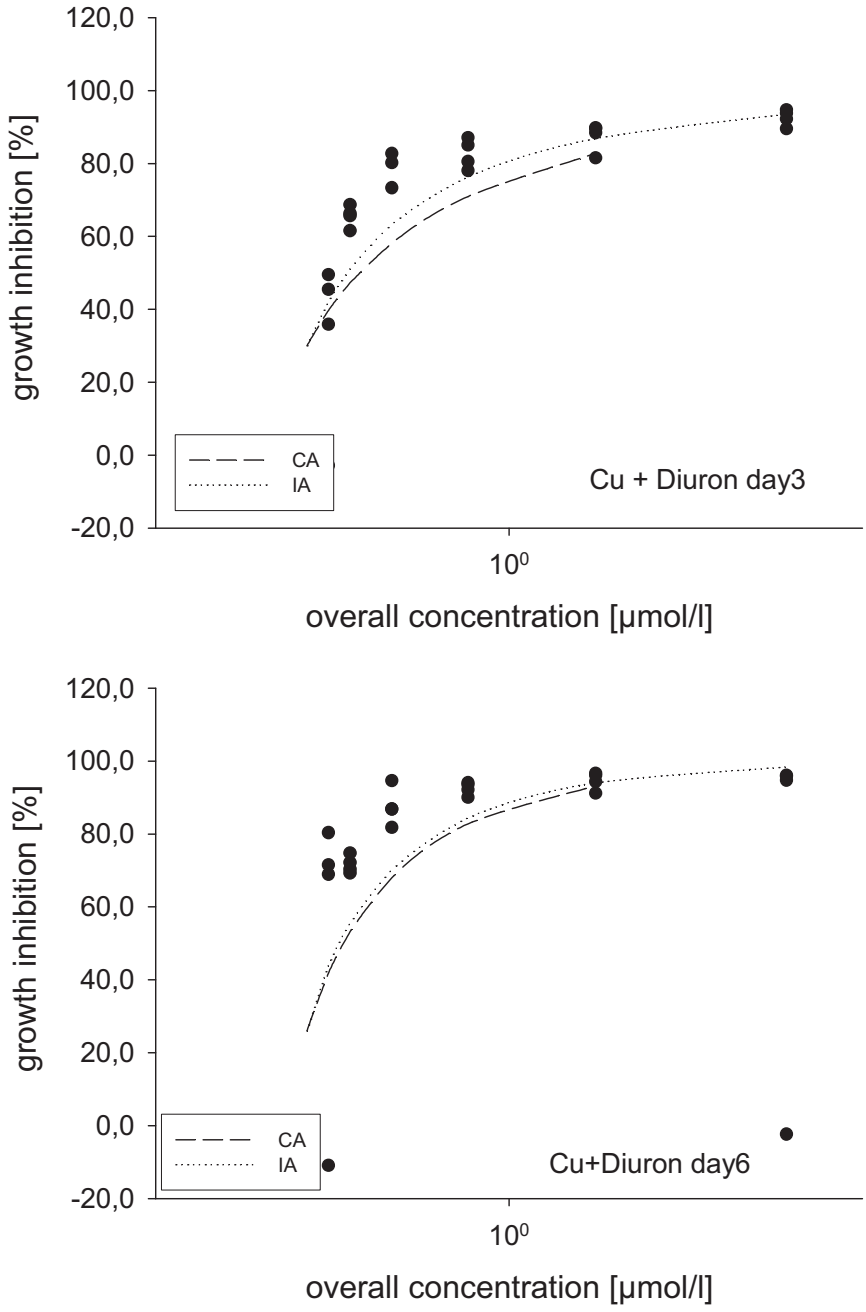


Figure 34: Prediction of the toxicity of two sequential exposure pulses of copper and Diuron.
The plants were pre-treated with copper for three days in a concentration causing approximately 30% growth inhibition (0,673 µmol/l). Subsequently the plants were transferred to a medium with various concentrations of Diuron and were exposed up to six days. The concentration is the overall concentration of Cu and Diuron the plants had been exposed to sequentially.

4 Discussion

4.1 Test conditions

Lemna minor is a plant that grows on the surface of waterbodies and it mainly grows two dimensionally. Thus, growth may not only be established by frond counting as proposed by the OECD and ISO test guideline(Organisation for Economic Co-operation and Development (OECD), 2006; International Organization for Standardization (ISO), 2006) but also by measuring the total area of the plant. Eberius et al. discussed the advantages of establishing the growth rate via the plant area. Standard deviations are minimized, confidence intervals are reduced and the test sensitivity is enhanced due to the individual measurement of the plant area at the beginning of the experiment. In comparison, the number of fronds may be identical but the area may vary and growth depends more on the plant area (photosynthetic active area) than on the frond number (Eberius et al., 2002). Additionally, in comparison with the frond counting method fewer plants are required and the experiments can be conducted in six well plates. The sensitivity of this test design is similar to the test design based on frond counting. The comparisons with EC-values based on frond counting found in the literature are similar to the EC-values based on frond area in this work of the studied herbicides. Hence, establishing the growth rate via total area should be favoured, as the required amount of plants as well as the amount of test volume and space for testing is reduced and the number of possible concurrent experiments is increased. Additionally, in the case of Alachlor, frond counting was not appropriate to establish the Alachlor-caused growth inhibition. Alachlor had an impact on the frond size. The plant developed dwarfish fronds. This observation is in agreement with other studies. An abnormal cell size of *Scendesmus vacuolatus* exposed to chloracetanilides (Vallotton et al., 2008) was observed. In this case the cell division failed and giant cells were the result. Similar observations were also made with *Chlamydomonas* (Fedtke, 1982). In the case of *Lemna minor* however the impact was the opposite. From these indications it can be presumed that the inhibition of the lipid biosynthesis has an impact on cell division. The exact cause for the development of dwarfish fronds however remains to be investigated. Also the fronds of heavy metal treated *Lemnas* were smaller as the control plants as shown in Figure 5 in the result chapter. Thus, the determination of the growth inhibition via frond area would have been more sensitive. However, more technical requirements such as a digital camera and an image editing program such as Photoshop as used in this work are needed to establish a growth rate via the total area of the plant. These requirements were not fulfilled at the beginning of this work and thus studies with the triazines and heavy metals were conducted

with the frond counting procedure. Furthermore, in the case of the metals, a higher quantity of plants was necessary to detect the metal uptake in the plants.

4.2 Single substance toxicity - how does time determine toxicity and which factors are important for the toxicity-time-relationship?

4.2.1 General discussion

This work focused on the MeoA and MoA and additionally in the case of metals on the uptake over time. These investigations only partly give an insight into the time dependency of toxicity. According to Rozman (Rozman 2000) the time dependency of toxicity is governed by toxicokinetics which includes uptake and elimination and toxicodynamics including injury and recovery. Focussing on the slower time-determining processes, these parameters can be further subdivided into *distribution*, *biotransformation* and *excretion* in the case of elimination and *adaption*, *repair* and *reversibility* in the case of recovery.

Though it was not possible to quantify to which extent recovery was due to either adaption, repair or reversibility based on the experimental results, it could be shown that recovery depends on how a substance binds to the site of action i.e. MeoA and what physiological impact a substance has i.e. MoA. For instance, in the case of the PS2 inhibitors, the unchanged toxicity over time indicated that there was no accumulative damage and that in accordance with the binding to the PS2 site (weak H-bonds) the MeoA is quickly reversible. Concluding from the visual recorded effects the secondary effect - oxidative stress - did either not come into play in the case of the PS2 inhibitors or was not so severe as to be seen by the naked eye. One may expect that a toxicant causing oxidative stress which is irreversible would lead to an accumulative damage and hence to an increasing toxicity. However *Lemna minor* showed a well-developed adaption to oxidative stress. Thus, in the case the PS2 inhibitors Ametryn, Prometon and Diuron, the PS1 inhibitor Paraquat and the metals investigated, the adaption superimposed the injury caused to such an extent that the damage did not accumulate and toxicity remained quite unchanged over time.

Apart from the introduced MeoAs and MoAs found in the literature also other effects are possible which may also influence the toxicity over time. However, assuming that these additional effects are more unspecific as for instance narcosis it may be concluded on the basis of the classification of Verhaar et al(1992) who state that the more unspecific the MoA the higher the effect concentrations, that these more unspecific MoA do not play such a great role in the concentrations tested here.

The time dependency of toxicity is also governed by the concentration i.e. the dose the organism is exposed to. Toxicokinetics and toxicodynamics are influenced by concentration. The change of the steepness of the concentration response curves over time gave an indication on how toxicity changed over time differently for different effect levels. This is also in agreement with the statement by Hayes (1975): At low effect levels, a sufficiently high rate of detoxification can negate the effects from extended exposure. This could be shown for the herbicides investigated but could not be confirmed by the metals nickel and zinc. If considering that plants have a well-developed homeostasis such as immobilisation by means of phytochelatines or organic acids, then this is surprising. One would expect due to the fact that lower excess metal concentrations can be better controlled than higher excess metal concentrations that the steepness of the concentration response curves would increase. Reasons for the decrease of the steepness of the concentration response may be that apart from the immobilisation of excess metal concentrations which may indeed operate better at lower concentrations, other aspects govern the toxicity. As discussed for nickel in detail one reason may be the saturation of the metals transporters at higher concentrations.

Toxicokinetics was only studied for the metals. The internal metal concentration could be easily measured via AAS. The uptake of the investigated herbicides could not be measured as simple as that. Options may have been C14 labelled herbicides. It can however be assumed that toxicokinetic only plays a minor role for lipophilic herbicides as the uptake is fast. Lipophilic, neutral herbicides which passively diffuse into plants rapidly reach equilibrium concentrations (Sterling, 1994). With the exception of Paraquat this is the case for all investigated herbicides. As uptake studies show a Michaelis-Menten-Kinetic a protein mediated active transport has been suggested for Paraquat (Hart et al. 1992). The same carrier as for putrescine has been proposed. Their study indicates that also the transport of Paraquat into the plant is fast.

As shown for the metals it is crucial how well a toxicant is taken in. The internal concentrations of copper, nickel and cadmium that caused a growth reduction of 50% were quite similar, despite their largely differing external EC_{50} values. Hence, the apparent different toxicities of these metals can be largely attributed to their different bioconcentration factors. Nevertheless, the comparable internal EC_{50} concentrations do not imply the same toxic potency of the different metals, as they show different distributions between the cell wall, the vacuole and the cytosol (Ernst, 1998). Not all the metal in the plant is necessarily bioactive, but the latter fraction can be expected to have the highest toxicological impact on

the plants. In order to gain more insight into where the metals taken up are distributed within the plant and in the plant cell, dyes which bind to free and hence bioactive metal-ions would be helpful. This is however beyond the scope of this work.

The increasing toxicity of zinc and nickel could not be assigned to an increasing internal concentration but must be attributed to a dynamic distribution within the plant. The distribution of metals is not static but a dynamic process, which means that the free available and bioactive metals and hence the toxicity of the incorporated metals can change over time. This has been shown by several studies. Phytochelatines are probably only a transient solution, as cadmium and copper phytochelatine complexes diminish after 7 to 14 days (Leopold et al., 1999; Kwan and Smith, 1990b) in plants. The formation of proline, which is presumably responsible for metal tolerance (Mehta and Gauer, 1999; Bassi and Sharma, 1993a; Bassi and Sharma, 1993b) is probably a short-term biochemical answer. About 24 hours after exposure to heavy metal begins, stress proline has diminished. These studies and the findings in this work clearly show that the metal toxicity over time is governed by a changing distribution within the plant over time. Hence the toxicity observed is not directly proportional to the internal metal concentration but is ruled by the distribution within the plant. Presumably the metal uptake is also not directly proportional to the external concentration, as the uptake of metals can be limited by the ion channels. Indications for a reduced uptake of heavy metals and a saturation of the transporters at higher concentration were observed for nickel and zinc. The change of toxicity over time was lower for higher effect concentrations than for smaller effect concentrations. The reason for this is presumably a saturation of the transporters at higher external concentrations. A confirmation of these presumptions and an additional insight into the time dependency of toxicity at different effect levels could be gained if the internal metal concentration within the plants were measured for the whole concentration range as the BCF is not necessarily the same for all concentrations. However this was beyond the scope of this work.

4.2.2 Herbicides

Triazines

A study with *Lemna perpusilla* and *Lemna major* gave EC_{50} values for Ametryn of 0,23 and 0,046µmol/l (Liu and Cedeno-Maldonado, 1974). These data lie within the same order of magnitude as the EC_{50} value of this work which is 0,085 µmol/l at day seven. So far, there are no studies with Prometon on *Lemna* so a comparison with the data on the toxicity of Prometon is not feasible. The visual recorded effects indicate no oxidative stress as reported by Hock and Fedtke (1995b). Reasons for this may be the absolute light intensity

(photooxidative stress is primarily observed in rather high light intensities) and the light adaptation of the plants. They were kept in constant light before the experiment, which typically results in a well-developed defence system against light-induced oxidative stress. Also, the time-independency of toxicity indicates that oxidative stress of triazine toxicity does not seem to play an important role under the conditions of this assay. No accumulative damage occurs. The binding of the s-triazines to PSII via non-covalent hydrogen bonds is reversible and thus the concentration that is actually bound to the receptor is dependent on the actual concentration of the chemical in the thylakoide membrane and consequently in the growth medium. As already discussed by Hayes et al. (1975) the dose time relation may vary for different effect levels. Due to the unchanging steepness of the concentration response curves of the triazines there is no change of the dose/time relationship over the whole range of the effect levels. Though the observed disintegration of the fronds at high effect level concentration indicates that the damage became more severe and that probably other effects came into operation apart from the interruption of the electron transport at the PSII site, this did not have an impact on the concentration response relationship and on the change of steepness over time. Additionally, even at high effect concentrations oxidative stress which would lead to a cumulative damage and thus to an increasing toxicity did not occur and was not observable as the fronds did not show necrosis and chlorosis.

Diuron

The EC_{50} value of this study (0.152 µmol/l) lies between the findings of (Okamura et al., 2003) with a 7d-EC_{50} of 0.129 µmol/l and Liu with an7d-EC_{50} value of 0.17 µmol/l (Liu and Cedeno-Maldonado, 1974). The findings are similar, though in this work, growth inhibition was based on frond area and not on frond number as in the work of Okamura et al. and Liu and Cedeno-Maldonado. Effects such as chlorosis caused by oxidative stress were not observed, although this may be due to the MeoA and its subsequent effects.

Concerning the EC_{50} value over time, similar observations were made for Diuron exposed plants as made for triazine exposed plants. The EC_{50} did change but without any trend. This change can rather be assigned to variability in the test results than to time-dependent change of toxicity. However, there was a slight increase of the steepness of the concentration response curve over time. The difference may be due to the other binding mechanism to the plastoquinone site or other additional effects. As in the case of the triazines, oxidative stress was not observable. Therefore oxidative stress as an additional effect is unlikely and hence the increase of the concentration response curve may either be due to other unknown additional

effects which gain more importance at higher concentrations or it can be due to the different binding mechanism of Diuron.

Paraquat

Like the plants exposed to heavy metals, Paraquat exposed *Lemna minor* showed chlorotic and necrotic fronds which is in agreement with the prior MeoA of Paraquat; oxidative stress caused by electron transport inhibition at the PSI site. The 7d-EC_{50} value (0.294µmol/l) as well as the 5d –EC_{50} value (0.321µmol/l) are similar to the finding of Fairchild (1997) who stated a 4d-EC_{50} value of 0.274µmol/l. There was a slight in increase in steepness and a shift of the concentration response curves to the low dose area. This indicates that the damage on the plants increases over time and that repair rate is slower than the rate of damage. Presumably, *Lemnas* exposed to high effect levels are more affected by accumulative damage as indicated by the increasing steepness of the concentration response curves over time.

Aclonifen

According to the literature, no studies have been done on the effect of Aclonifen on *Lemna minor*. Thus, a comparison of the EC-values is not possible. Aclonifen exposed plants showed a concentration-dependent loss of pigments. This is in agreement with the MoA, the inhibition of the formation of chlorophyll. However, growth as an integral endpoint is not immediately affected. Though there is a visual difference as shown in Figure 4, there is no difference concerning the growth rate. Thus, it can be concluded, that growth is not a very sensitive endpoint in the case of Aclonifen. Aclonifen has a delayed effect on the growth rate which becomes apparent on the third day of the experiment. Based on the standard test procedure this delayed effect leads to an underestimation of the effect due to the average growth rate over the whole test duration. Hence, 100% growth inhibition could not be achieved according to the standard test procedures (International Organization for Standardization (ISO), 2006; Organisation for Economic Co-operation and Development (OECD), 2006). Presumably other results with observed effects up to 100% may have been observable if using another endpoint such as chlorophyll content. If simply looking at the change of steepness of the concentration response curves over time one may overlook the change of toxicity over time as the steepness remained quite unchanged. However, the shift of the whole concentration response curve over time indicates that the delay of toxicity had the same importance at every effect level. The effect on chlorophyll reduction may be concentration-dependent as the visual observations have indicated. Additional effects like the formation of PPIX and the formation of harmful singulett oxygen as described by Hock and Fedtke may also occur. One may assume based on

the findings of Hayes (1975) low effect concentrations are more easily negated than high effect concentrations and that the lag phase would be different for different effect levels. However this was not the case. This indicates that oxidative damage, which is not quickly reversible and should lead to cumulative damage to some extent, was probably not the main cause of growth reduction.

Alachlor

In the case of Alachlor, establishing the growth rate by frond counting would underestimate the effect on growth due to the development of dwarfish fronds. Fairchild and co workers name EC_{50} values which are higher than the EC_{50} values found in this study. After a four day exposure the EC_{50} values found by Fairchild are 0,734µmol/l (1997) and 1,786 µmol/l (1998) whereas in this work the EC_{50} values are 0,314 µmol/l after a three day exposure and 0,179µmol/l after a five day exposure. These data are based on the frond number and this may be the cause for the differences. An abnormal frond size was not reported by Fairchild.

There was an increase in toxicity over time which was effect level dependent. This is in agreement with the findings of Chang et al. (1985). Hence, this inhibition is probably the cause for the time dependence. This is also supported by the postulated MeoA of the chloracetanilides, covalently binding to sulfhydroxyl-groups of enzymes (Fuerst, 1987). This binding is irreversible and thus the damage accumulates and the toxicity increases.

Similarly to the observations made with Aclonifen, an effect of 100% could not be achieved if growth were averaged over the whole test duration. This is, as in the case of Aclonifen, caused by the delayed effect on growth.

4.2.3 Metals*

The observed necrotic and chlorotic fronds exposed to the metals investigated zinc, copper, nickel and cadmium is in agreement with the described effect of heavy metals, oxidative stress caused directly or indirectly. The cause of the smaller fronds, which was observed for all metals investigated is unclear but may be linked the inhibition of enzymes which influences the frond size. Naumann et al. (2007) investigated ten heavy metals on *Lemna minor*. Apart from the endpoint growth inhibition they also investigated the chlorophyll and the carotinoides. Especially nickel had an impact on the pigment content in their study which is in agreement with the observed pale fronds of nickel exposed plants in this work. They

* Parts of this chapter have already been published elsewhere; see: Drost W, Matzke M, Backhaus T (2007): Heavy metal toxicity to *Lemna* minor: studies on the time-dependence of growth inhibition and the recovery after exposure. Chemosphere 67(1): 36-43

could not show a correlation between ligand affinity and toxicity. Hence, the toxic mechanism is more subtle than the simple interaction with proteins and enzymes. A metal with a high ligand affinity referring to ligands such as proteins with oxygen sulphur and nitrogen-ligands may however also be better sequestered by metallothionines and thus may be better controlled.

Cadmium

The EC_{50} values recorded in this study proved to be comparable to previous studies with *Lemna*. An EC_{50} of 1,5 µmol/l was determined by Kwan and Smith (1991) for cadmium after ten days incubation, while an EC_{50} of 2µmol/l was found after 7 days exposure in the present study. Naumann et al. (2007) also used the Steinberg medium as in this work. This is an important aspect as the toxicity of metals is greatly influenced by other cations due to their influence on the uptake of the metals (Kwan and Smith, 1991). Naumann et al. found an EC_{50} of 3µmol/l based on frond counting after seven days of exposure.

There was neither considerable change of steepness over time or a change of the EC_{50} value over time, which indicates that the damage caused by cadmium was not cumulative. As the visual results showed that cadmium apparently caused oxidative stress in *Lemna minor*. This MoA is highly unlikely to be quickly reversible. However *Lemna minor* has presumably a well-developed defence and repair system referring to oxidative stress. This presumption can be supported by the observations made with the PS2 inhibitors. The plants were kept in constant light before the experiment, which typically results in a well-developed defence system against light-induced oxidative stress. Even though as an unessential metal a cadmium homeostasis may seem more unlikely, Kwan and Smith (1990a) showed that a major proportion of accumulated cadmium in *Lemna minor* is bound to proteins which immobilises cadmium and Grill et al. (1985) showed that the phytochelatine syntheses are induced not only by essential metals such as copper and zinc but also by cadmium.

With an average BCF of more than 1300 cadmium had the highest BCF of all the metals examined. This might indicate a co-uptake with Calcium, an essential macro-nutrient. This kind of interaction between calcium and cadmium has already been suggested by Kwan and Smith, (1991). The uptake of cadmium is influenced by pH and by the calcium concentration as studies have shown (Skowronski et al., 1991; Kwan and Smith, 1991). Hence, the BCF is different for other compositions of test medium. The internal cadmium concentration significantly decreased over time. This is in contrast to the findings of Kwan and Smith (1991) who found the uptake kinetics of cadmium into *Lemna minor* to be linear over time

and the internal cadmium concentration to constantly increase. Reasons for this deviation may be the use of a different external cadmium concentration, which was approximately half the concentration used in this work and a quarter of the strength of the Steinberg medium. As essential nutrients; copper, nickel and zinc were present in the controls. However cadmium was also found in the control plants which must be assigned to a contamination either from the bi-distilled water or from the salts used as nutrients.

Copper

Ince et al. (1999) and Lakatos et al. (1993) reported an EC_{50} for copper of 23,6 µmol/l at day seven whereas Teisseire et al. (1998) recorded an EC_{50} value of 2.52 µmol/l after the same exposure duration. With 9,682 µmol/l based on frond counting, the EC_{50} of the presented study falls within this span. Naumann et al. (2007) recorded an EC_{50} of 2,7µmol/l after a seven day exposure. However in a time span of approximately two years *Lemna minor* became more sensitive to copper in this work. In an experiments conducted later on, a EC_{50} of 1,789 µmol/l was determined, which is more in accordance with the small EC_{50} values of Teisseire and Naumann. The fact that this value is based on frond area and not on frond counting may explain differences in the derived EC values but cannot fully explain the increase of sensitivity of the plants. Though copper caused smaller fronds, the downsizing of the fronds was not that dramatic as to explain the tenfold increase of sensitivity. *Lemna minor* were obtained from two different laboratories, experiments conducted on the basis of frond counting were *Lemnas* from a different source than tests conducted on the basis of frond area. However, prior to changing the *Lemna*, tests with Atrazine and copper had shown that the sensitivity was similar (data not shown). The fact that the plants used for the experiments which were based on frond area were grown in another growth medium in the other laboratory may be a reason. The plants may have adapted to the growth medium used in this work with the result of their becoming more sensitive.

As in the case of cadmium, there was no change of toxicity over time over the whole effect range. This is probably also due to a well-developed defence and repair system. As copper is an essential metal *Lemna minor* are likely to have a well-developed regulation system to keep up the copper homeostasis.

Nickel

The EC_{50} value for nickel of this work is considerably higher compared to another study with *Lemna minor*, though the test conditions were identical. Naumann et al. (2007) found an EC_{50} of 6,2 µmol/l which is approximately 1/10 of the EC_{50} value of this work. There may be

various reasons for this deviation, such as different adaption of the plants to nickel but presumably not to metals in general as the comparison of the EC-values of the other metals studied shows.

As opposed to the metals cadmium and copper, nickel toxicity increased over time especially in the concentration range causing effects up to 50% growth inhibition. This caused in contrast to the findings with the investigated herbicides, a decrease in the steepness of the concentration response curve over time. This is unusual as the opposite would be assumed if considering that plants have a well-developed defence system against metal intoxication (Tsuji et al., 2002; Kwan and Smith, 1990a; Grill et al., 1987). Hence one would expect that due to a better regulation of lower metal concentrations than higher concentrations, the steepness of the concentration response curve would increase as the toxicity increases especially at higher effect concentrations. Considering these observations it must be concluded that there are other aspects apart from defence, control and repair that govern the toxicity/time relationship. Though the uptake of the metals over time has been investigated it is not however clear whether the observed uptake kinetics at concentrations causing approximately 50% growth inhibition is the same for other effect concentrations. Metals are actively transported into the organism. The internal metal concentration is not necessarily directly proportional to the external concentration but can also be governed by the number of available metal transporters. Mallick et al. (1996) investigated the uptake kinetics of nickel, chrome and zinc and found that the uptake of these metals followed a Michaelis-Menten kinetic. Changes in the uptake rate were only observed up to a certain external concentration showing the saturation of the transport system. The saturation of the nickel-transporters may be a reason why toxicity did not increase to such an extent at higher concentrations.

Zinc

For zinc, Ince et al. (1999) reported an EC_{50} value of 147 µmol/l, which is slightly higher (by a factor of 3,2) than the EC_{50} of 46,1 µmol/l that is reported in this work. This might be due to the different growth medium used which greatly influences the toxicity of metals. As in the case of nickel also the EC_{50} value found by Naumann et al. (2007) for zinc were lower than in this work. Naumann et al. found an EC_{50} value of 10,4 µmol/l which is one fourth of the EC-value found in this work. The detected EC values are four times the arithmetic mean environmental concentration of zinc (1,692 µmol/l) referred to the EC_{25}.

The same observations were made concerning the steepness of the concentrations response curves over time as were made for nickel. Unexpectedly the increase of toxicity over time was

especially pronounced in the lower effect region. Reasons may be same as were discussed for nickel - the active transport of metal cations may determine the steepness of the concentration-response curve and the transport system may be saturated at higher concentrations.

The increase of toxicity could not be explained by an increase of the internal concentration of zinc. This may be due to cumulative damage. Zinc strongly induces the formation of phytochelatines but it is not a prominent ligand as shown in maize and radish (Souza and Rauser, 2003) and hence not well controllable by phytochelatines. Excess zinc concentrations may be regulated via the formation of zinc phytate as it was found in *Lemna minor* and *Deschampsia caespitosa* (Van Steveninck et al., 1990; Van Steveninck et al., 1987). Phytic acid, myo-inositol hexakisphosphate normally functions as storage of minerals in plant reproduction. This mechanism may function for shorter exposure durations or lower concentrations but may have failed under the experimental conditions in this work. Additionally phosphate is generally not very abundant in plants and is probably even less abundant in natural waters compared to growth medium with a high concentration of nutrients.

4.2.4 Predicting toxicity over time-

Is Haber's rule observable and are Haber's rule or its derivations an appropriate tool to describe toxicity-time relationships?

According to Rozman (2000), the experiments of this study should show simple or more complex c•t=k relationships. Indeed if toxicity increased over time, the toxicity-time relationship could be well described by these equations. Hence with the exception of Diuron and Prometon which did not show increased toxicity on *Lemna minor* and thus indicate the limitations of this approach, Haber's law and its deviations gave a good description of the iso-effective curves.

Experimental requirements to use Haber's rule and its derivations

Coming from the branch of human toxicology Rozman and Doull (2001b) extensively discussed under which circumstances i.e. what test design is necessary to observe Haber's rule or deviations of it. If kinetic as well as the dynamic half life is short as it is the case for inhalation narcotics the c•t=k relation can only be observed, if exposure occurs continuously. In this case toxicity is rather dose-dependant than time-dependant. The exposure frequency is crucial if the kinetic or dynamic half life is intermediate relative to the duration of the experiment. Then recovery can occur and Haber's law is not observable if the exposure

frequency is longer than the elimination or recovery time. If due to an irreversible binding to the site of action no recovery can occur, than toxicity is especially time-dependant. In order to assess the hazard of a substance on humans, tests are conducted with rats or other mammals. Due to the test design in these cases, the exposure to a toxicant occurs in pulses if the substance tested has to be administered for instance by an injection. Hence in the hazard assessment of substances on humans, the exposure conditions are more complex than they are in standard aquatic ecotoxicology tests where exposure occurs continuously and kinetic steady state is experiment driven.

Comparison with an alternative empiric approach

Facing the fact that there are more acute toxicity data than chronic toxicity data the acute to chronic ratio (ACR) has been discussed as one method to extrapolate from short-term toxicity to long-term toxicity (Ahlers et al., 2006;Länge et al., 2004). Acute EC_{50} values are simply divided with chronic NOEC or the lowest observed effect concentration (LOEC) values to achieve an ACR. Länge et al. (2004) analysed acute to chronic ratios (ACR) for various types of substances using the ECETOC Aquatic Toxicity (EAT) database. They calculated the ACR and ranked the values according to increasing values and calculated the 90%-ile of the resulting distributions und suggest to use this 90%-ile ACR value as an default extrapolation factor. However especially metals, organometals and pesticides show highly variable ACRs and were thus excluded. Junghans et al. (2008) have discussed the ACR as an extrapolation method further into detail.

The role of the power term γ

Miller et al. (2000) discuss the simple $c \bullet t = k$ relation as a special case in a family of power term curves. Thus they suggest using a general form: $c^{\alpha} \bullet t^{\beta} = k$. This form of equation gives the ability to distinguish whether the toxicity is more concentration- or time-dependant. If c and β equal 1, dose and time are equally important in determining toxicity and Haber's law applies. α and β may also be combined to γ or x which than leads to the equation described by Bliss. As time is a multidimensional parameter, the power term γ was used for the parameter time, though it does not make a difference whether the power term used for the concentration or time. The power term γ gave a good insight into the toxicity-time relationship of the investigated substances and the weight of time. There is clearly a link between reversibility and the parameter time. Substances with a similar MoA and the same reversibility may have a similar power term. This should be further investigated. Ametryn reversibly blocks the PS2 system. The power terms for all investigated effect levels were smaller than one, which

indicates that concentration and not time governed the toxicity of Ametryn on *Lemna minor*. The observations made and the calculated power term γ confirm that oxidative stress also an indirect effect of PS2 inhibitors and more irreversible than the weak H-bonds of PS2 inhibitors to the site of action, did either not come into play or *Lemna minor* possesses a good defence system. Also the recovery experiments conducted with the PS2 inhibitors, which are going to be discussed in detail later on, indicate that the effect of PS2 inhibition quickly reversible even for high effect concentrations. The power terms for Aclonifen and especially for Alachlor were larger than one, which indicates that time played a more important role to the toxicity of these substances than the concentration. Again, these findings are underpinned by the findings made with the recovery experiments. The inhibition of the biolipid syntheses (Alachlor) as well as the inhibition of chlorophyll syntheses (Aclonifen) affected the plants to such an extent, that *Lemna minor* did not fully recover within the duration of the recovery experiment. Hence, damage can accumulate over time, which means that the observed effect reflecting the toxicity of a tested substance is governed by time. Paraquat is also a photosynthesis inhibitor but at the PS1 site and it causes oxidative stress directly. Nevertheless the power terms found for Paraquat were smaller than one or one. Oxidative stress was apparent as the fronds showed necrotic and chlorotic fronds, however the findings indicate that *Lemna minor* have a good defence and repair mechanism under the circumstances of the test which leads to the result that the damage was less cumulative. This may also be the reason why the toxicity of the metals investigated based on the calculated power terms which were smaller than one in the case for cadmium, copper and nickel, was less time-determined than concentration-determined. Zinc was an exception as in agreement with the observations made. The toxicity of zinc was clearly time-determined.

The different time dependencies for different effect levels

In agreement with the findings of Hayes (1975) the dose/time relation of a substance varied for different effect levels. This shows that is not only important to consider toxicity and time but also to regard different effect levels over time. Altenburger and Greco recently suggested combining time- and concentration-dependent toxicities into a global concentration-time effect-model (Altenburger and Greco, 2009). They did however not consider different time-dependencies for different effect levels but estimated a time-dependent estimate of the median potency (EC_{50}). This work shows that in the case of the herbicides the power term was larger for the higher effect level of 75% growth inhibition than for the lower effect level of 25% growth inhibition, showing that time becomes a more governing factor of toxicity at higher effect levels. At low effect levels, a sufficiently high rate of detoxification can negate the

effects from extended exposure. Hayes et al. (1975) showed this in a study with hydrocyanic acid and thus showed the limitations of Haber's rule which is based on the assumption that toxicity always increases over time. However in contrast, the metals studied showed the opposite results. According to the power terms, time was a more governing factor at the lower effect level. This is in accordance with the decreasing steepness of the concentration response curves of nickel and zinc, which was due to the increasing toxicity especially in the case of the lower effects.

Is DEBtox an appropriate tool to deal with toxicity over time?

Comparison with the approach of Haber

Compared to the approach just discussed, DEBtox is a mechanistic model. Toxicodynamics is not considered in the calculation the model implies that the effect is completely reversible and thus toxicity is determined by the concentration and hence toxicokinetic. DEBtox only requires data which are derived from standardized tests.

The Haber equation and its derivation are empirical models and thus cannot be discussed concerning the mechanisms which govern the toxicity over time. In contrast to the DEBtox model this approach does not make any assumptions concerning toxicokinetics or toxicodynamics. Though simple, this approach nevertheless gives a good description of toxicity over time and the power term γ can be used to describe what governs the toxicity of a substance, whether the toxicity is more determined by the dose or time. As has been discussed, it can be used as an extrapolation-tool for environmental risk assessment in order to extrapolate from short exposure data to long exposure. However, in contrast to the time independent NEC value derived by DEBtox these values are time-dependent.

Unlike the approach propagated by Rozman and Doull, the aim of the DEBtox model is not a description of the toxicity over time but to derive an NEC which is time-independent. As DEBtox is a mechanistic model it makes assumptions concerning the mechanism governing the toxicity. Whether these assumptions are too simplistic or wrong due to the properties of the substance and the organism will be discussed in the following. The two approaches compared have different aims and different advantages and disadvantages. The approach propagated by Rozman and Doull: Haber's law and its deviations are simple and easily understandable and applicable to ecotoxicological data as discussed above. As an empirical model its aim is the description of toxicity over time and additionally it is a useful tool to extrapolate from acute toxicity data. It has however the disadvantage that the data or

extrapolations are always linked to a certain time. This is in contrast to the NEC derived by DEBtox which is time independent. The aim DEBtox is the derivation of a time-independent threshold value as an alternative to the currently used NOEC. The mechanistic model does however make assumptions which do not apply to all substances.

Discussion of the mechanistic assumptions of DEBtox and comparison with other mechanistic models

The assumption made by the authors of DEBtox is in accordance with the critical body residue (CBR) approach, which is developed for chemicals with a completely reversible effect (McCarty, 1986). From its origins the CBR model was designed on the basis of studies with organic compounds with no specific but narcotic effects. However, the effect of toxicants may not be reversible or only slowly reversible leading to accumulative damage. Verhaar, Legierse and co-workers developed the critical target occupation model (CTO) model, which quantifies the effect through the integral of the target occupation over time (Verhaar et al., 1999; Legierse et al., 1999). For their studies they used organophosphorous pesticides which covalently bind to the acetylcholine-esterase. This model assesses the overall toxicity, if the binding to a receptor is irreversible and there is no de novo receptor synthesis and the toxicant binds covalently to the active site. The CTO is associated with a critical time-integrated concentration, the so called critical area under the curve (CAUC). In contrast to the CBR and the DEBtox model, the CTO-model assumes that the critical threshold changes i.e. decreases over time.

However these model regard two extremes, most chemicals lie between these two cases. Lee et al. (2002) developed a Damage Assessment Model (DAM) in order to predict the time course of toxicity of polycyclic aromatic hydrocarbons (PAHs). This model is based on the assumption that the damage is proportional to the body residue and that recovery is proportional to the cumulative damage. Compared to the CBR and CAUC approach the DAM can provide an estimation of recovery which lies in between no recovery at all, with a recovery rate of zero, and immediate recovery with a recovery rate of ∞.

Considering the aspect that complete reversibility may be under-estimative, Jager and Kooijman extended the DEBtox model adding receptor kinetics (Jager and Kooijman, 2005). The same data which were also used to develop the CTO model (Legierse et al., 1999; Verhaar et al., 1999) and additional data of the investigation of acetylcholine-esterase inhibitors were used. Different from the CTO model the receptor model can deal with irreversible and reversible interactions like the DAM model. It is different from the DAM

model as damage is not considered as first order but it is calculated using hazard modelling. From their investigations and the results the authors conclude that the standard DEBtox model is nevertheless useful for hazard assessment as the NEC estimated from the extended model is basically identical to the NEC estimated with the standard DEBtox model. However, the DEBtox model has found some criticism if it comes to scenarios where the exposure is not continuous but fluctuates over time (Ashauer et al., 2006a). This will be discussed later on.

Is the DEBtox-derived NEC a good alternative to the NOEC?

The major aim of the DEBtox model is not so much to produce a description of the toxicity over time but to derive an NEC which is time independent. It has been greatly criticized among scientists and regulators (Chapman and Caldwell, 1996; De Bruijn et al., 1996) that the NOEC is used for the derivation of a Predicted No Effect Concentration (PNEC) in regulation. The latter is derived from available data such as the NOEC or EC_{50} and applied with a safety factor which can vary between 1-1000 depending on the amount and quality of data. The NOEC is always one of the test concentrations and thus strongly depends on the test design. It is the highest test concentration that gives no significant deviations from a control (Bartlett et al., 1974; Bringmann and Kühn, 1980). If the conduction of an experiment has been sloppy this results in a higher NOEC. Additionally, the term 'No Observed Effect Concentration' blurs the fact that indeed NOEC concentration can cause an effect. Depending on the test design, the NOEC value may even be larger than the EC_{50} value. This is certainly not desirable. Alternatives have been suggested (Chapman and Caldwell, 1996; Hoekstra and Van Ewijk, 1993). One of them is the use of the NEC (Kooijman and Bedaux, 1996a; Kooijman and Bedaux, 1996b; Kooijman Bedaux, 1996c; Kooijman, 1981). Additional data or modifications of the test guidelines are not requested for the use of the DEBtox-tool. Hence the NEC can be derived from data which are already available and according to Jager et al. (2006) can use the data even more efficiently. As a model parameter this value is not one of the test concentrations and it has a standard deviation. Additionally compared to any of the other alternatives it is time independent. The DEBtox-tool is indeed user-friendly as the creators state. No extensive knowledge is necessary to use the DEBtox tool for the calculation of NEC values; however the use of 'black box' generated data may be unappealing.

Considering the aspects discussed above the NEC seems a good alternative to the NOEC. If considering mixtures even a mixture of substances in their NEC-concentrations may nevertheless less lead to observable effects. Studies showed that even mixtures in their EC1 ratios or NOEC-ratios showed effects (Breitholz et al., 2008; Faust et al., 2003; Silva et al.,

2002; Altenburger et al., 2000; Backhaus et al., 1997). According to Baas et al. (2007, 2010) substances may share the same NEC so even if single substances occur in concentration smaller than their NEC the mixture of the substances can exert an effect. Presumably any suggested concentration of a single substance greater than zero which is used in order to identify a concentration under which a hazard is unlikely to occur is hampered if mixture toxicity is taken into account. Hence, the question arises whether there is any concentration which can be labelled with the property of causing no effect independent of how this concentration has been derived as substances do not occur singly but in mixtures in the environment. Additionally as the NEC is based on standardised toxicity tests, the NEC is always restricted by the shortcomings of toxicity tests. Effects may remain undetected due to the test design. For example, if lethality is used as an endpoint sublethal effects are overseen. Using reproduction as an endpoint may neglect such effects that do not affect reproduction in the single species testsystem but can have an impact if referring to an ecosystem with predators. The drawbacks of the determination of thresholds under which no effect is supposed to occur has been extensively discussed by Grimme et al. (1990). The naming blurs the fact that substances occurring in the environment can nevertheless pose a risk to the environment even though their environmental concentration is lower than the identified threshold under which, according to the definition, no effects occur. Though the risk may be small it nevertheless is not zero. By using ECx values instead, this may give a clear statement that the derivation of a threshold does not mean that there is no risk for the environment if the environmental concentration is smaller. What effect concentrations should however be used? If small effect concentrations are used what is small? For the derivation of small EC values tests must be designed differently. A redesign of the test-protocols may be time and cost consuming and so far data are too scarce to simply neglect old NOEC data as de Bruijn and van Leeuwen state (1996).

4.3 Variable exposure to single substances- How does the toxicity change and what determines toxicity over time?

4.3.1 Pulsed exposure -What determines the recovery potential?

General discussion

If and to which degree an organism recovers from the exposure to a pulse of a toxicant depends on various aspects. As long as the substance is present at the site of action it will cause a toxic effect. Thus the level of detoxification and /or elimination of the toxicant play a vital role. Another aspect which determines the recovery is the degree of toxic impact and thus the recovery from a damage, which depends on the concentration as well as the MoA and MeoA. As shown in the cases of the PSII inhibitors and Alachlor, the issue how the toxicant binds to the site of action is crucial. Week H-bonds are easily reversible covalent bonds are not. Recovery also depends on the repair rate which depends on what damage has been caused. This work also shows that the concentration *Lemna minor* had been exposed to can determine the recovery potential. As already mentioned in the discussion on toxicity over time, at low effect levels a sufficiently high rate of detoxification can negate the effects from extended exposure. Lipophily can in part be one but not stand alone factor governing the recovery potential. Hence, if referring to substance properties, the recovery potential depends on the quantity of the damage as well as the quality of the damage.

The organism and the question how well can it deal with a pulsed toxic impact are additionally important. As a study on *Daphnia magna* with insect growth regulators has shown, the life stage of the organism and its susceptibility to the toxicant governs the impact of the substance and thus the recovery (Hosmer et al., 1998). Life stage dependent toxicities were also observed for fungicides on *Daphnia magna* (Hassold, 2010). As different life stages are not distinguishable for *Lemna minor* this issue could not be investigated in this work. On an ecosystem level the recovery may gain another importance. An ecosystem may recover concerning its functioning, however may have moved from one stable state to another as van Straalen (1992) discussed.

Herbicides

The effects of s-triazines as well as Diuron on the growth of *L. minor* were easily reversible. This is in accordance with the findings of Cedergreen et al., who investigated the recovery potential of the PSII inhibitor terbuthylazine on *Lemna minor* (Cedergreen et al., 2005). The duration of the pulse in their study was three hours. In order to observe equivalent effects at such a short exposure duration concentration were tenfold or hundredfold higher than the

long-term effect concentrations. In another work *Lemna gibba* also showed a comparable recovery after a five day exposure to Atrazine (Hughes JS et al., 1988). Also other plants showed fast recovery after a pulsed exposure to PSII inhibitors (Macinnis and Ralph, 2003; Kersting and Wijngaarden, 1999; Van Geest et al., 1999). These results can be traced back to the underlying biochemical mechanism of s-triazine action as already discussed. The inhibition of the photosynthetic electron transport is per se non-lethal to the organism. Only the growth rate is reduced – even down to zero if sufficiently high concentrations of an s-triazine were present – because the photosynthetic energy production is inhibited. Furthermore, the binding of the s-triazines to PSII via non-covalent hydrogen bonds is reversible and thus the concentration that is actually bound to the receptor is dependent on the actual concentration of the chemical in the thylacoide membrane and consequently in the growth medium. As soon as the concentration in the growth medium decreases, so does the internal concentration. Consequently, the inhibition of the photosynthetic electron transport ceases and the organisms start growing again. Oxidative stress, also an indirect effect of the PSII inhibitors did not seem to play an important role under the conditions of the assay as has already been discussed in the chapter for single substance toxicity.

Cedergreen et al. (2005) concluded that focus should rather be put onto to herbicides with other MoA than PSII inhibition. They additionally studied the recovery potential of *Lemna minor* after an exposure to ALS inhibitors, microtubule assembly inhibitors and PSI inhibitors. Recovery from the cell deviation affecting substances as well as from the ALS inhibitors occurred slowly. The plants did however not show any growth reduction after a three hour pulse to the PSI inhibitor diquat. This result is in contrast with the findings of this work. Paraquat exposed plants showed only slow recovery. The plants in the work of Cedergreen et al. were however only exposed to a hundredfold higher concentration of the EC10 value obtained for a long-term exposure. This effect level is comparatively low to the effect level of 50% growth-inhibition and as already Hayes (1975) has mentioned the quantity of damage can also play a role as smaller quantities of damage may be more easily negated. In the study of Cedergreen at al. (2005), investigations were made as to whether recovery is governed by the lipophily of the herbicides or governed by their MoA/MeoA. The uptake rate as well as the level of detoxification and /or elimination of the toxicant plays a vital role in the recovery. Lipophily is an important aspect for the two factors uptake and elimination whereas detoxification depends on the organism. Cedergreen et al. (2005) concluded that recovery depends on the toxicant as well as its lipophily and that both factors are important. This is in agreement with the findings of this work. Alachlor, Ametryn, Diuron and Prometon have

similar $logK_{OW}$-values of about three but showed different recovery patterns. Plants exposed to the PSII inhibitors showed a fast recovery whereas plants exposed to the lipid synthesis inhibitor Alachlor recovered slowly. Having exactly the same MeoA, Ametryn and Prometon may have been a good basis to investigate the importance of lipophily concerning recovery. However, as both substances also have very similar $logK_{OW}$ values this research question remains unanswered.

The MeoA of Alachlor is not fully understood but it has been assumed that Alachlor may covalently bind to the thiol groups of proteins (Hock et al., 1995a). Hence in this case the recovery can rather be assigned to the MeoA than to the lipophily. The study with Alachlor shows the importance of how the substance is bound to the active site. In agreement with a study with *Daphnia magna* the recovery from organophosphorous insecticides was bad, due to a covalent bond to the active site, whereas recovery occurred fast if the bond were reversible as was the case for carbamate insecticides (Kallander et al., 1997). In this case if the substance were covalently bound, the aspect of lipophily may be of minor importance for elimination.

Aclonifen has a $logK_{OW}$ of four and therefore is slightly more lipophilic. This may in part explain the slow recovery after an exposure pulse to Aclonifen. However the MeoA, the inhibition of the chlorophyll syntheses is not quickly reversible. An enzyme is blocked which makes it necessary to produce de novo enzymes. Additionally oxidative stress may occur. However, equally to the PSII inhibitors necrotic fronds were not observed.

Paraquat is positively charged. Hence according to the lipophily hypothesis the plants should rapidly recover. The observations however support that the induction of oxidative stress, is a more important factor influencing the recovery rate in this case.

The recovery potential was also studied for different concentrations in the case of the triazines Diuron and Alachlor. In agreement with the findings of the concentration-dependency of single substance toxicity over time, recovery was concentration-dependent if the MeoA was not quickly reversible. In the case of Alachlor, toxicity over time became more time-dependent at higher effect concentrations indicating irreversible and cumulative damage. In accordance with these observations, plants exposed to a concentration causing 50% growth-inhibition did not recover but did show slow recovery if the plants had been exposed to a concentration causing 30% growth inhibition. In the case of the PSII inhibiting herbicides, the concentration the plants had been exposed to did not have an influence on the recovery

potential even if the plants had been incubated with extraordinary high concentrations in the case of the triazines. This is also in accordance with the toxicity over time observations. There was no change of the concentration response curves over time.

Metals[*]

Even seven days after transferring *Lemna minor* into uncontaminated medium the zinc pre-exposed plants still showed a severely reduced growth rate. Copper and cadmium pre-exposed *Lemna* showed growth rates close to control level after three days recovery, while nickel pre-exposed *Lemnas* actually reached control levels.

As expected, the internal concentrations of all metals decreased during the recovery phase. In principle, two processes might be responsible for the decrease in metal concentrations: (a) active or passive excretion of the metal from the plant and (b) the "dilution" of the internal metal concentration due to an increase in total biomass (growth). Though efflux transporters in plants have been found for zinc, copper, cadmium and nickel (Hall and Williams, 2003) a significant decrease was only detectable for copper (factor 2) and nickel (factor 3), while the total amount of zinc and cadmium in the plants remained constant.

The rapid decrease of nickel concentrations is most likely responsible for the rapid recovery of the plants after nickel exposure. Added with the comparatively low bioconcentration factor, this might argue for an active efflux system in *Lemna minor*. This would be in concordance with the findings of Hall and Williams (2003).

In any case, the rapid decrease of internal concentrations during the recovery demonstrates that nickel – and also copper, which shows a similar pattern - is not irreversibly bound to any cell structure such as the cell wall.

On the other hand, the dynamics of the internal concentrations of cadmium and zinc did not point to any efflux from the exposed plants. In contrast the data showed that the decrease in cadmium and zinc concentrations was completely due to the increase in biomass. This could be due to the binding of cadmium to various polypeptides and the subsequent transport into the vacuole (Kwan and Smith, 1990b; Vögeli-Lange and Wagner, 1990; Weigel and Jäger, 1980). Zinc and cadmium were also shown to be sequestrated in the vacuole by organic acids, such as malate and oxalate (Krotz et al., 1989; Mathys, 1977).

[*] Parts of this chapter have already been published elsewhere; see: Drost W, Matzke M, Backhaus T (2007): Heavy metal toxicity to *Lemna* minor: studies on the time-dependence of growth inhibition and the recovery after exposure. Chemosphere 67(1): 36-43

Taking the non-efflux of zinc together with the extremely flat concentration-response curve, this might offer an explanation for the non-recovery after the zinc-exposure. Due to biomass increase, the internal concentration decreased only a little. Zinc had an extremely flat concentration-response curve and therefore this slight decrease was not sufficient to lower the internal concentration to non-toxic levels. In contrast, cadmium had a slightly steeper concentration-response curve and consequently the dilution of internal concentrations due to growth seemed to lower the internal concentrations sufficiently to allow growth recovery of the plants.

This hypothesis however assumes a one-compartment model, i.e. an even distribution of the metals within the plants and their mobility during growth. Another line of reasoning for explaining the non-recovery after a zinc exposure would be that zinc mainly binds to and is specifically toxic for meristematic tissues of the *Lemna*s. This could lead to locally lethal, i.e. non-reversible effects on this tissue and thus preventing the subsequent growth of new fronds – even after the external exposure ended. But as visual observations indicated chlorosis occurred evenly in all tissues of the plants, this hypothesis might be considered somewhat unlikely. All in all, further studies are needed to collect more details on the distribution of the metals in the different parts of the plants and possible different toxic effects on the different tissues.

4.3.2 Fluctuating exposure-How does toxicity evolve?

Discussion of the investigated cases with the substances Alachlor, copper and Diuron

The emission of hazardous substance into the environment may occur in pulses. Depending on the exposure duration and the time of the recovery phase and the recovery potential, this may lead to a cumulative effect or an apparent lessening in toxic response due to adaption. In this work pulses were conducted sub-sequentially. The plants were exposed to a concentration causing 30% growth inhibition and were then immediately exposed to a whole concentration response curve. If no recovery occurs at all, there is no adaption and the toxicity of the substance is constant over time, this would lead to upshift of the concentration response curve with the lowest response starting at 30% growth-inhibition.

As the results show this is not the case for any of the investigated substances. In the case of Alachlor the sensitivity increased. The plants became too sensitive to the concentrations tested in order to show a concentration-dependant response. This became especially apparent at day six where the data points were all at the top of the single substance concentration-response

without pre-treatment. This is in accordance with the assumed MeoA, the alkylisation of thiol groups of proteins (Fuerst, 1987). As the toxicity of Alachlor increases over time this explains why the response was even higher than 30% growth inhibition. Due to the pre-treatment the plants had already been exposed to Alachlor for three days. Hence effectively three days of exposure have to be added. However one would expect that those plants which were subsequently exposed to a lower concentration then the concentration of the pre-treatment would show recovery to some extent as has been shown in the recovery experiments. However the recovery potential decreased to such an extent that this could not be observed.

In the case of copper, adaption occurred. This is indicated by the response observed for the lower concentrations. The plants showed a higher growth rate than the control plants if referred to the pre-treated control plants. The plants recovered better if exposed to small doses of copper. Cause for this adaption may be the increase of phytochelatines or proline, which leads to a more effective scavenging of the internal metal; or the induction of antioxidative responses may have reduced the vulnerability of the plants to oxidative stress. Additionally, *Lemna minor* are likely to have a well-developed regulation system to keep up the copper homeostasis as copper is an essential micronutrient.

The study with fluctuating Diuron concentration was not fully in accordance with the findings of other studies not shown in this work with *Lemna minor* and PSII inhibiting triazines (Drost et al., 2003). The pre-exposure to Diuron did have some though small effect on the sensitivity of the plants towards Diuron at day three. In the case of the triazines Ametryn and Prometon there had been no impact on the sensitivity of the plants. All three herbicides are PSII inhibitors. The plants showed very good recovery from three day pulses to the three PS inhibitors. In accordance with the MeoA of Diuron and the observed recovery potential one would have expected similar results. The reason for the difference may be a different binding to the site of action as has been postulated by Tietjen (1991).

Comparison of observations of other studies

The study with *Lemna minor* and fluctuating concentrations of Alachlor, copper or Diuron shows that the damage is either cumulative, the plants can adapt or the plants show an increase of sensitivity only over a short time due to fast recovery. Conclusions of other authors concerning repeated pulses are contradictory. Hosmer et al. (1998) studied fenoxycarb on *Daphnia magna* and concluded that pulses with intermission are less toxic than continuous exposure, whereas Buhl et al. (1993) showed that *Daphnia magna* were more sensitive to repeated pulses of the bromoxynil formulation Buctril than to a continuous exposure to this

substance. However, the sensitivity of organisms to pulsed exposure is dependent on a combination of concentration, pulse duration and interval as Naddy et al. (2001; 2000) have shown. Toxicokinetic and toxicodynamic play a vital role. If no adaption occurs and the intermission between the pulses correspond to the toxicokinetics of a substance that means the second pulse occurs after the first substance has been eliminated from the organism, the impact of the second pulse will only depend on the toxicodynamics i.e. the recovery from the first pulse. In the case of concentration-dependent toxicants with a fast recovery rate, the first pulse will not have an impact on the sensitivity. In contrast in the case of substances with a MoA with slow recovery or no recovery at all, the first pulse will indeed have an impact on the sensitivity. If the intermission is shorter than the toxicokinetics of a substance, the impact on the sensitivity is determined by toxicokinetics as well as toxicodynamics. The second case can be assumed for this work as the pulses were subsequently followed by a second pulse.

If looking at mesocosms, repeated pulses may lead to the selection of the more hardy individuals and may change the species composition (Van Straalen et al., 1992). On the other hand, Kersting and Wijngaarden (1999) observed a decrease of sensitivity of the tested mesocosm exposed to the PSII inhibitor Linuron, which was rather caused by adaption of the organisms as the species composition remained unchanged (Van Geest et al., 1999). However the authors conclude that with other exposure regimes, the composition of the mesocosm may have changed. Hence if considering ecosystems, the impact of pulsed or fluctuating exposure does additionally depend on the composition of the ecosystem apart from concentration, pulse duration and interval. This was however beyond the scope of this work.

Discussion of possible appropriate approaches to assess the toxicity of substances that occur in pulses

Haber's law or the derivation as a power term has been used to assess the toxicity of a substance that occurs in pulses. The assessment is based on a time-weighted average concentration. However this is only applicable for pulsed exposure - the concentration remains constant and the exposure is intermittent. This was however not the case in this work. The concentration was not constant but changed over time. As Ashauer et al. (2006a) concluded in a review on various approaches which consider toxicity over time this approach does have some limitations as the relationship between effect and dose factor is not defined and linearity is assumed. Toxicokinetics and toxicodynamics both play an important role concerning pulsed or fluctuating exposure. Especially toxicodynamics becomes very important as the recovery potential determines whether a pre-exposure makes the organism more sensitive to the subsequent second exposure. As discussed for single substance toxicity

over time, there are several approaches which consider toxicity over time and make assumptions concerning toxicokinetics and toxicodynamics.

According to the CBR approach which is the basis for DEBtox toxicity it only depends on concentration i.e. toxicokinetics and the impact is instantaneously completely reversible. If regarding death as the endpoint, this means according to the model that a dead individual will come back to life, which is of course not true. In contrast the CTO approach assumes that the effect is completely irreversible. Both approaches make assumptions concerning the recovery rate either being 0 or ∞. In the case of pulsed exposure the CBR approach would only take into account the elimination rate regardless of the time to recover from the caused damage. If the next pulse occurs after a duration which is longer than the elimination rate the pre-exposure history would be neglected. In the case of the CTO approach any pre-exposure history would be taken into account regardless whether there was an intermission and regardless of the duration of the intermission. The DAM approach can provide an estimation of recovery which lies in between no recovery at all, and immediate recovery. The damage is proportional to the internal concentration and the repair rate is proportional to the damage. Death occurs if damage reaches a certain threshold. However in this model it is not possible to establish a negative hazard rate as it is the case for other endpoints. This may seem counterintuitive as repair and recovery is included in the model.

Coming to the conclusion that none of the existing models are appropriate for pulsed or fluctuating exposure, Ashauer et al. (2006a) have suggested a modification of the DAM model which also allows a negative hazard rate. Ashauer et al. conducted experiments with *Gammerus pulex* which were exposed to pulses of pentachlorphenol, carabaryl and chlorpyrifos (Ashauer et al., 2006b; Ashauer et al., 2007c) and determined the uptake and elimination rates of these substances (Ashauer et al., 2006b). They developed a new model called Threshold Damage Model (TDM) which takes into account toxicokinetics as well as toxicodynamics. The other models discussed (CBR, CTO and DAM) are special forms of this model (Ashauer and Brown, 2008). However apart from recovery experiments in order to use this model it is necessary to know uptake and elimination-rates. These may be determined by using QSAR and the log KOW values. However so far a QSAR for *Lemna minor* has not been developed and measuring uptake and eliminations rates of the investigated herbicides was beyond the scope of this work

4.4 Mixtures under simple and complex exposure conditions- how does mixture toxicity change over time and is it predictable?

4.4.1 Mixtures with a constant composition-

General discussion

The results of his work show that both concepts CA and IA give an overall good prediction of the mixture toxicity for all four mixtures investigated though the mixtures with the substances Alachlor and copper and Alachlor and Diuron were underestimated in some parts of the concentration response relationship. The mixture toxicity of the copper-Diuron mixture was slightly underestimated for the middle effect area but overall the predictions fitted well with the observed toxicity. The predicted mixture toxicity with all three substances was in accordance with the experimental findings. On the basis of these findings, it can be concluded that the concepts prove to be a good predictive tool for simultaneous mixtures.

Due to their MoA the investigated substances are independently acting mixture components. Thus one may conclude that IA should make better predictions of the mixture toxicity than CA. Indeed IA made a better assessment of the mixture toxicity which is also confirmed by other works (Faust et al., 2003; Backhaus et al., 2000; Hermens and Leeuwangh, 1982). However other findings generally showed that CA overestimated mixture toxicity and hence this concept has been suggested as a general solution to assess mixture toxicity on the basis of the precautionary principle (Faust et al., 2003; Faust et al., 2000). In this work IA generally estimated smaller effect-concentrations than CA and hence on the basis of these findings IA should be used as a general solution for the assessment of mixture toxicity. However, the mixtures only consisted of two or three components and the predictions of the two concepts were very similar. Additionally, in the work of Junghans (2006) it was shown that the possible deviation between the predictions made by the concepts depends on the number of mixture components. For a binary mixture the possible deviation is very small. Hence on the basis of the results of this work it cannot be concluded that IA would be a better overall approach to predict mixture toxicity.

Discussion of the observed and predicted toxicity of the Alachlor, Copper and Diuron mixtures

The comparison of the observed and predicted toxicity of the copper and Alachlor mixture clearly showed an underprediction of the mixture toxicity. Hence, the observations made are not in line with the prediction of the mixture toxicity which indicates synergistic effects according to Placket and Hewlett (1948). The interaction between Alachlor and copper may

be the influence on the uptake of one of the components or the change of the physiological action. Though the MeoA of Alachlor is not fully understood it has been postulated that Alachlor may react with the nucleophilic thiol-group (-SH) of proteins releasing chloride or an aryloxy residual. To overcome a metal intoxication or to control an excess metal concentration, metals are immobilized by means of chelatation to cystein-rich phytochelatines (Tsuji et al., 2002; Kwan and Smith, 1990a; Grill et al., 1987). These cystein rich polypeptides supply many thiol groups for binding metals but may also interact with Alachlor. Interestingly the interaction between Alachlor and copper was the opposite if *Lemna minor* were exposed sequentially to these two substances. Antagonistic effects were observed if Alachlor was the first substance. Alachlor may trigger a physiological response which makes the plant less vulnerable to an excess copper concentration. On the other hand copper does not seem to trigger a physiological response which makes the plants less vulnerable to Alachlor. Apart from a physiological response within the plant, an influence on the uptake can be the cause. The different exposure patterns lead to the possibility that the substances can interact and can thus change the uptake if occurring simultaneously or not if occurring sequentially. This may be the explanation why either synergistic or antagonistic effects are observed for different exposure scenarios of the combination of Alachlor and copper.

In the case of the combination of Alachlor and Diuron it is unlikely that the under-prediction of the mixture toxicity is a synergistic effect. The assessment of the mixture effect fitted well with the observed response for the lowest dose and the higher mixture concentrations but underestimated the toxicity for the middle concentration area. Hence there was not an overall consistent under-prediction for certain concentrations as was the case for the combination of Alachlor and copper. Additionally according to the MoAs of these substances, an interaction as is possible in the case of Alachlor and copper is unlikely.

The mixture of copper and Diuron was well predicted. Though the observations had shown that there is an interaction between Alachlor and copper, this did not come into play if all three substances were combined. This may be due to the increased number of components. Interaction between substances can be ruled out if the number of substances in a mixture is increased (Warne and Hawker 1995).

Mixture toxicity over time

The assessment of the mixture toxicity is based on the single substance concentration response relationship. The assessment of the mixture toxicity was predicted for day three and day six for which concentration response curve were available. As the curves are fitted to the

data the toxicity-time relationship is inherent in the fit of the data, the concentration response curve. As the prediction of mixture is based on single substance toxicity the assessment of mixture toxicity faces the same shortcomings as single substance toxicity assessment: toxicity studies generally only referre to certain time points and neglect the time course of toxicity. Equally to the case of single substance exposure, the toxicity-time relationship can be described by the Haber equation and its derivations. As the results show the mixture specific exponent γ differs from the single substance exponents but is determined by its mixture components and their single substance toxicity-time relationship. It is however on the basis of the results of this work not clear how the substance specific exponents combine in a mixture. To elucidate this issue further work is necessary. Alternatively, it may be a more straightforward approach to assess mixture toxicity over time by using the single substance specific exponents and combining the effect level specific exponents to an overall time-concentration-response relationship. A similar approach has been suggested by Altenburger and Greco (2009). They suggested predicting mixture toxicity over time with a global single substance concentration-time-effect model. They use a time-dependent estimate of the EC_{50} to describe the toxicity-time-relationship.

Baas and co-workers approach the problem of mixture toxicity over time using the concepts CA and IA and combining them with the mechanistic model DEBtox. Initially this approach was developed for binary metal mixtures (Baas et al.2007) and then extended for mixtures that can have any number of components (Baas et al. 2009a). The following assumptions are made: In accordance with the DEBtox model only toxicokinetic is considered to influence toxicity over time. Simple one compartment first order kinetic is assumed. Substances, that have the same MoA, share the same NEC in accordance with the CA-concept whereas substances with a different MoA each have their individual NEC. That means a mixture of substances with the same MoA would cause an effect if the concentration of each component equals the NEC whereas no effect would occur if the substances have different MoAs. Referring to the findings of this work toxicity over time cannot be solely assigned to toxicokinetic. Toxicodynamic is equally important. Additionally, it is questionable whether an organism can equally well cancel out the effect of a substance if exposed to a mixture as if exposed to the substance singly even though all substances may have different MoAs. That means: Is the NEC of a substance really unaffected in the presence of other substances with other MoAs especially if it based on integral endpoints such as death or reproduction? This question however so far remains unanswered. The effects of mixtures with substances, which are according to their MoA independently acting, in concentration which are assumed to have

no effect have yet not been studied. The comparison of experimental data of the mixtures and predicted mixture toxicity seems to prove the accuracy of the model (Baas et al. 2007; Baas et al 2009b; Baas et al. 2010). However, in the case of the binary metal mixtures (Baas et al. 2007) it is unclear whether this is indeed due to the model or due to the extensive data amount used. In contrast the results of this work hint that metal-toxicity is neither due to simple first order kinetics nor to toxicokinetic alone. Only very few data were used predicting the mixture toxicity of PAHs (Baas et al. 2010). Toxicokinetic parameters were assessed using $\log K_{OW}$ values. As PAHs act as narcotics their toxicity is indeed mainly governed by their uptake i.e. toxicokinetic. Hence, the model and its mechanistic implications are sufficient in the case of narcotic PAHs. Baas et al. (2009b) also made predictions of daphnid survival after in situ one week exposure to complex mixtures of polluted surface waters. The survival of daphnids was extrapolated from toxicity data established for shorter time periods than one week. Indeed, the concepts CA and IA are not suitable to extrapolate to other points in time as the authors criticise. However, it is not shown to which extent the concepts would have failed to predict the survival of the daphnids.

4.4.2 Mixtures with a fluctuating composition

Discussion of the investigated cases

Single pulse recovery experiments show a correlation between the MoA and MeoA and the recovery potential. However, on the basis of the recovery potential conclusions of a pre-exposure on the sensitivity of the plant to a second substance could not always be drawn. Plants pre-exposed to Diuron or copper became more sensitive to Alachlor though *Lemna minor* showed a fast recovery after a single pulse of Diuron and copper. *Lemna* showed growth rates close to control level after 3 days recovery. On the other hand Alachlor pre-exposed plants showed a decrease in sensitivity towards copper although the plants had shown a slow recovery. The decrease in sensitivity can be due to a physiological response of the plants as a consequence of the Alachlor exposure, which makes the plant less vulnerable to copper. It has been postulated that Alachlor may react with the nucleophilic thiol-group (-SH) of proteins releasing chloride or an aryloxy residual (Fuerst, 1987; Chang et al.1985). This reaction may trigger a physiological response such as the formation of new proteins with cystein groups. However it is unclear why this phenomenon was not observed when copper was the first substance the plants were exposed to. To control an excess metal concentration, metals are immobilized by means of chelatation to cystein-rich phytochelatines (Grill et al., 1987; Tsuji et al., 2002; Kwan and Smith, 1990a). These cystein-rich polypeptides supply many thiol groups for binding metals but may also interact with Alachlor. However the

physiological response after an Alachlor pulse seems more effective for scavenging excess copper than the physiological response if exposed to copper, the formation of cystein-rich proteins. As already discussed the interaction between Alachlor and copper was the opposite if *Lemna minor* were exposed simultaneously to these two substances. Synergistic instead of antagonistic effects were observed. Synergistic or antagonistic effects are due to toxicokinetic or toxicodynamic interaction. Toxicokinetic interactions involve the alteration of metabolism or influence on the uptake whereas toxicodynamic interactions involve a physiological alteration making the organism more or less sensitive. An influence on the uptake can be excluded if the exposure is sequential. This interaction can however occur if the combination of the substances is simultaneous. This may be the reason for the different effects observed for different exposure scenarios as in the case of Alachlor and copper. The antagonistic effects observed if an exposure to Alachlor is followed by an exposure to copper do however indicate that there is also a toxicodynamic i.e. physiological interaction within the plant. As the case of the combination of Alachlor and copper shows, synergistic as well as antagonistic effects can be observed for the same combination of substances depending on the exposure, which can either be simultaneous or sequential. If an interaction is due to an influence on uptake this effect can only occur for simultaneous mixtures.

Apart from the sequence, which is important, the duration of the intermission between pulses is important. A sufficient decline of the damage which is determined by the recovery rate will not contribute to the effect of a subsequent pulse. The recovery rate is determined by toxicokinetics and toxicodynamics. Without an intermission however, as was the case in this work, the pre-exposure does have an impact due to no full recovery. As already discussed, conclusions on the sensitivity to subsequent exposure could not necessarily be drawn on the basis of the recovery experiments. Presumably the recovery potential from the first substance may be influenced by the second substance, decreasing the recovery rate and increasing the sensitivity. Considering this aspect, single substance recovery experiments may only in part be a good basis for assessing the mixture effect of sequential mixtures especially if the duration between the pulses is shorter than the recovery rate.

Comparison with observation of other studies

As shown, the sequence of substances is crucial for the overall effect. Different results were observed for the same combination of substances but with different sequences. This is in agreement with other works. Macinnis-Ng et al. (2004) found that the sequence copper followed by the PSII inhibitor Irgarol was more toxic to sea grass than the sequence Irgarol

followed by copper. Ashauer et al. also concluded from their results that the sequence of the exposure matters (Ashauer et al., 2007a). They investigated the effects of Carbaryl and Chlorpyrifos on *Gammarus Pulex*. These two substances are insecticides, which inhibit the acetylcholine-esterase either by covalently and irreversibly binding (Chlorpyrifos) or reversibly binding (Carbaryl) to the enzyme. In accordance with the results of this work the recovery potential is an important aspect. However, though single pulse recovery experiments showed a correlation between MoA and recovery potential conclusions of a pre-exposure on the sensitivity of the plant to a second substance could not always be drawn on the basis of the recovery potential in this work.

Based on their findings Ashauer et al. (2007b) developed the Threshold Damage Model (TDM), which takes into account toxicokinetics as well as toxicodynamics. On the basis of the measured uptake and elimination rates of each substance singly, they made a prediction of the overall effect of a sequential combination of Carbaryl and Chlorpyrifos and vice versa by calculating the internal concentration and the caused damage. The experiments were conducted with a recovery period in-between the pulses, which is in contrast to this work where one pulse was subsequently followed by second pulse. The recovery period between the pulses was either shorter than the established recovery time from a single pulse (Chlorpyrifos) or longer than the recovery time from a single pulse. Ashauer et al. conclude that the TDM is applicable to other exposure regimes. However, considering that the results of this work indicate that the recovery potential from the first substance may be influenced by the second substance decreasing the recovery rate, overlapping pulses may lead to a different result than expected by the model. The two concepts CA and IA were not implemented into this model and how the TDM relates to the concepts still needs to be investigated (personal communication). The model showed good performance compared to the experimental data. So far the TDM is only applicable for tests which consider death as an endpoint. A modification of the TDM for sub-lethal effects is on the schedule (personal communication) but so far not available. Hence, the TDM is not applicable to the data of this work.

Discussion of the applicability of the concepts CA and IA to predict combination effects of sequential mixtures

The concepts are applicable within limits to predict combination effects. If considering that substances do not necessarily occur simultaneously in the environment and that there is recovery to some extent, the two concepts may be used as a worst-case assessment of combination effects of a fluctuating exposure scenario as the concepts do not take recovery into account. Conceptually CA may be applied if there is not a complete depuration and an

internal mixture occurs. The concept of IA can be regarded as a sequence of different toxicants one after another. Compared to CA, IA is independent of the depuration and considers combination effects, which may occur depending on the MoA even after depuration, which may indeed be the case for sequential exposures. Considering these issues, IA may be regarded as a better approach for predicting the effects of a combination. However, only binary combinations have been investigated in this work. Hence, the differences of the assessment of the combination effect were too small to come to a conclusion concerning their predictive power. Both approaches did not over-assess the combination effects of the binary combinations as assumed and therefore do not serve as a worst-case prediction tool of the effects of a combination of sequential mixtures. The concepts did however give good estimation of the combination effects as the results show.

Discussion of possible alternative approaches

Apart from this study other studies have also shown: sequence matters (Ashauer et al., 2007a; Macinnis and Ralph, 2004) and the duration of the intermission between pulses is also important. These aspects are determined by the individual toxicokinetics and toxicodynamics of each substance. However, toxicokinetics and toxicodynamics as discussed above are not considered in the concepts CA and IA. A first step combining toxicokinetics and toxicodynamics with the two concepts IA and CA has been conducted by Lee and Landrum (2006). They developed the Multi-Component Damage Assessment Model (MDAM). The aim of the work of Lee and Landrum is to find reasons for synergism and antagonism i.e. toxicodynamic and toxicokinetic interaction and therefore is somewhat different from the aim of this work. Nevertheless, this approach might be applicable to more complex combinations of different substances. However apart from the requirement of additional laboratory data to describe toxicokinetics and toxicodynamics this approach as well as the TDM has been developed for a test system with the endpoint death and therefore is not applicable to the data of this work.

4.5 Implication for current risk assessment procedures

So far risk assessment has regarded toxicity over time only insufficiently. Based on the findings of this work Haber's law or derivations from may serve as a good tool in environmental hazard assessment to describe and extrapolate toxicity over time. The power term γ may be used as a descriptor for the toxicity-time relationship. As the results of this work show, the power term γ gives a good insight into the toxicity-time relationship of the investigated substances and the weight of time. The Haber equation may serve as default description of the development of toxicity over time. Nevertheless, a modification of Haber's law (c^n x t =k) is only considered for time scaling in the human health hazard assessment as suggested in the guidance on the information requirements and chemical safety assessment for the implementation of REACH (European Chemicals Agency, 2008) but not in the equivalent document for environmental hazard assessment (European Chemicals Agency, 2008).

Though the discussion about the use of the NOEC is long lasting even new guidelines such as the guidance documents on information requirements under REACH (Guidance on information requirements: Hazard Assessment), which would have given the opportunity for alternatives nevertheless consider the NOEC. The generation and application of NOECs should be reconsidered for various reasons. The sloppier a toxicity test has been conducted the larger a NOEC value will become. A NOEC is unlike a benchmark value not a fixed value and can be far beyond a concentration not causing an effect and their use as input data for mixture toxicity assessment is questionable (Kortenkamp et al. 2009). Additionally a NOEC is always linked to the test duration from the test it has been derived from. Conceptually the NEC is a good alternative: a time independent threshold and a model parameter with a standard deviation. However the NEC is built on mechanistic assumptions that are contradictory to the findings of this work. Toxicokinetic alone is not sufficient. Toxicodynamic also needs to be taken into account. Therefore, if a time-independent threshold is to be introduced its extrapolation should, unlike the NEC, be based on toxikokinetic and toxicodynamic. Additionally, regardless how a threshold concentration is derived, it is questionable whether the label 'no effect' can be used with any certainty due to shortcomings of standardized toxicity tests such as single species test but multispecies/ecosystem reality, overseen effects and the occurrence of mixtures. Instead ECX values should be used also in regard to the data input requirements for mixture assessment. It should be proved that a toxic effect does not exceed a certain limit which has been set beforehand thus reverting the burden of proof (Hoekstra and Van Ewijk, 1993). This maximum tolerable concentration could be based on extrapolation to prolonged exposure and

thus would be time-independent. Of course, the question arises where the limit should be set and what effect concentration is small enough to be tolerated.

This work emphasise the necessity to include recovery studies in an ecological hazard assessment. Recovery experiments are only taken into account in the context of placing of plant protection products on the market so far. Recovery experiments provide additional information about the toxicity properties of a substance which might be essential for a more realistic environmental risk assessment and which can be gained from suitable adaptations of standard biotests. This might not add much information in the case of a fast, acute MeoA and for which the plants have a demonstrated high capacity for recovery, such as the PSII-inhibiting herbicides (Drost et al., 2003; Gustavson et al., 2003). But for substances such as for instance zinc only a more flexible exposure regime, also in standard bioassays, will allow to discover and analyse the implications of a highly dynamic toxicity pattern for their potential environmental hazard.

Mixtures matter and science provides a tool to reliably assess mixtures toxicities. However, regulation mainly focuses on single substances and mixtures are only considered in some parts of the community legislation such as the EU Regulation on classification, labelling and packaging of substances and mixtures (Council of the European Communities 2008). Lately however mixture mixtures have gained political interest. In December 2009 the council of environment ministers of the EU have concluded that mixtures should be taken into account and that further action is needed in the field of chemicals policy. The Council has invited the Commission to scrutinize this issue concerning the necessity of modifications in legislation and guidelines in order to appropriately address the risk of mixtures. In this context a report "State of the Art Report on Mixture Toxicity" which examined 21 directives and regulations has been prepared (Kortenkamp et al. 2009).

How to modify legislation and guidelines in order to appropriately address the risk of mixtures is not a simple quest. In order to implement the risk assessment of mixtures several directives and regulations need to be considered. Intentional mixtures with a certain purpose such as preparations of chemicals and products containing chemicals can be taken into account by the product-oriented legislations. Complex exposure situations which consider mixtures with substances related to different legislations are however beyond the scope of a product-oriented legislation. This may make it necessary to establish an overall regulatory framework. Media related regulations such as the WFD are better suited to take complex exposure scenarios of mixtures in the environment into account (Kortenkamp et al, 2009).

In order to assess mixture toxicity it is necessary to know its composition. Chemicals with a wide dispersive use or a high potential to be released into the environment due to its uses is more likely to be found in a mixture than chemicals restricted to a few uses, and a low potential to be released into the environment. These properties may give some orientation which chemicals are likely to be found in a mixture. REACH will eventually provide more information on how chemicals are used. REACH obligates manufacturers and importers as well as downstream users to communicate the risks of chemicals and the intended uses along the supply chain. Samples from the environment can give additional information on what kind of mixtures are to be expected and whether there are 'typical environmental mixtures'. It is however questionable whether all occurring mixtures can be identified. Therefore, as a pragmatic approach a mixture assessment factor has been discussed. The question is how large does this factor need to be to accurately take the occurrence of mixtures into account? Are the already existing assessment factors sufficient? These questions need to be investigated.

The prediction of mixtures toxicity depends on single substance toxicity data. This, of course, requires their availability. In the past chemicals have been brought onto the market and were used barely having any information on their hazards and risks. To overcome this information gap REACH was introduced 2007. With the claim "no data no market" REACH aims at closing this information gap. REACH will gradually lead to more data on toxicity of substances. The database IUCLID (International Uniform ChemicaL Information Database) which captures, stores and maintains data on hazard properties of chemicals supplies the data necessary to conduct mixture toxicity assessment.

Mixtures under complex exposure situations are yet another and maybe even greater but worthwhile challenge because they require additional information on pulse length, intensity, pulse frequency and the sequence of the different substances. The results of studies which take complex time patterns and exposure regimes into account may help to refine the risk assessment in the future for instance of crop protection and to indentify pulse sequences that may be less harmful to the environment than other use patterns.

4.6 Conclusion

Not only the dose/concentration of substance determines its toxicity but time. This work shows that toxicity over time under simple and complex exposure patterns is determined by several parameters. Toxicity over time depends on how a substance binds to the site of action i.e. MeoA and what physiological impact a substance has i.e. MoA and whether the damage cumulates. As the uptake and elimination of herbicides is fast toxicokinetic may only play a minor role for the toxicity-time-relationship. In the case of metals the assumption of a single compartment first order toxicokinetic is not sufficient. The changing toxicity over time may rather be attributed to a dynamic distribution of free available and bioactive metals within the plant than a changing internal concentration. Additionally, the toxicity-time-relationship is concentration-dependent. At low effect levels, a sufficiently high rate of detoxification can negate the effects from extended exposure. As metals are actively transported into plants, the transporter and its concentration dependent capacity additionally play a role. Hence, it is important to consider toxicity and time but also to regard different effect levels over time. As an empiric approach Haber's law or derivations from it give a good description of the toxicity-time relationship. In order to get a whole picture of the time-dependency of toxicity of a substance, the power term γ for different effect level can offer a good basis.

In accordance with the findings single substance toxicity over time under continuous exposure conditions, the concentration and effect level as well as the quality of damage are important factors that estimate the recovery potential after single substance pulses. Substances with a slow reversible MoA and MeoA and a time-dependent toxicity show a slow concentration-dependent recovery potential, whereas substances with a quickly reversible MoA and MeoA show a concentration-independent fast recovery potential. Additionally, recovery is due to a decrease of the internal concentration due to efflux or dilution via growth and increase of biomass as shown for the metals.

It is important to consider toxicokinetics and toxicodynamics when regarding toxicity over time. Mechanistic models which only consider toxicokinetics and make assumptions concerning the toxicodynamics are insufficient. Models which take toxicokinetic as well as toxicodynamic into account are TDM and the MDAM. Both approaches however also require additional laboratory data on uptake which are normally not considered in standard tests.

The exposure to single substance with fluctuating concentrations can lead to cumulating damage, adaption or plants show an increase of sensitivity only over a short time due to fast recovery. Pulse length, intensity, timing and pulse frequency are important parameters if

regarding fluctuating exposures. If the intermission between two pulses is in accordance with the toxicokinetics of the first substance the increase of sensitivity to the second pulse will depend on the toxicodynamics i.e. the recovery potential. Hence, the relation between the intermission and toxicokinetics and toxicodynamics are important to consider. Additionally, if combining different substances, the sequence of substances is crucial for the overall effect. Toxicokinetics and toxicodynamics and the sequence are not considered by the concepts CA and IA. Nevertheless, based on the findings of this work the concepts CA and IA are applicable within limits to predict combination effects under complex exposure situations.

4.6.1 Perspective

Haber's law or derivations from it give a good description of the toxicity-time relationship. The power term γ gives an insight into the weight of time. Substances with a similar MoA and MeoA and the same reversibility may have a similar power term. Due to differing repair and efflux systems of organisms and differing sensitivity the power term γ may vary for organisms. These issues should be further investigated.

Also an important issue is the timing of the pulses. If regarding time from the organism perspective life stages of organisms have a different susceptibility to the toxicant which governs the impact of the substance and thus the recovery (Hosmer et al., 1998). As different life stages are not distinguishable for *Lemna minor* this issue could not be investigated in this work but may be investigated with other test organisms such as daphnia.

Complex exposure situations are determined by pulse length, intensity, timing and pulse frequency and sequence. This work has investigated two substances occurring in one pulse each but should also be investigated for multiple pulses. A promising study on *Daphnia magna* has been conducted (Hassold, 2010). It is also important not only to investigate subsequent pulses but pulses with varying intermissions in order to elucidate the relation between duration of intermission and toxicokinetics and toxicodynamics. The work of Nathalie Valloton (Vallotton, 2008) has investigated this issue on algae using pulse sequences with varying intermissions as they are also found in the aquatic environment due to the seasonal use of crop protection. The possible variations are numerous and possibly lead to numerous different results. In order to take complex exposure situations into account in risk assessment and to deduce possible general rules this issue should rather be investigated systematically than investigating individual cases.

The tests were conducted with a single species test system but how does an ecosystem react on complex exposure? If regarding ecosystems the impact of pulsed or fluctuating exposure additionally depends on the composition of the ecosystem as well as the pattern of exposure. An ecosystem may recover when considering its functioning, however it may have moved from one stable state to another as van Straalen et al. discussed (Van Straalen et al., 1992) and hence may lead to a different response than single organism tests have shown.

It is important to consider toxicokinetic as well as toxicodynamic. Two models have been developed which either take the concepts CA and IA as well as toxicokinetics and toxicodynamics into account (MDAM) or assess combination effects on the basis of single substance studies on toxicokinetics and toxicodynamics (TDM). So far these models only deal with test systems which use the irrevocable death as the endpoint. Growth-inhibition however has a different recovery pattern which is not taken into account in these approaches. Hence future research should focus on the extension of these models to other endpoints than death.

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Annexe

Single substance concentration response curves

Herbicides

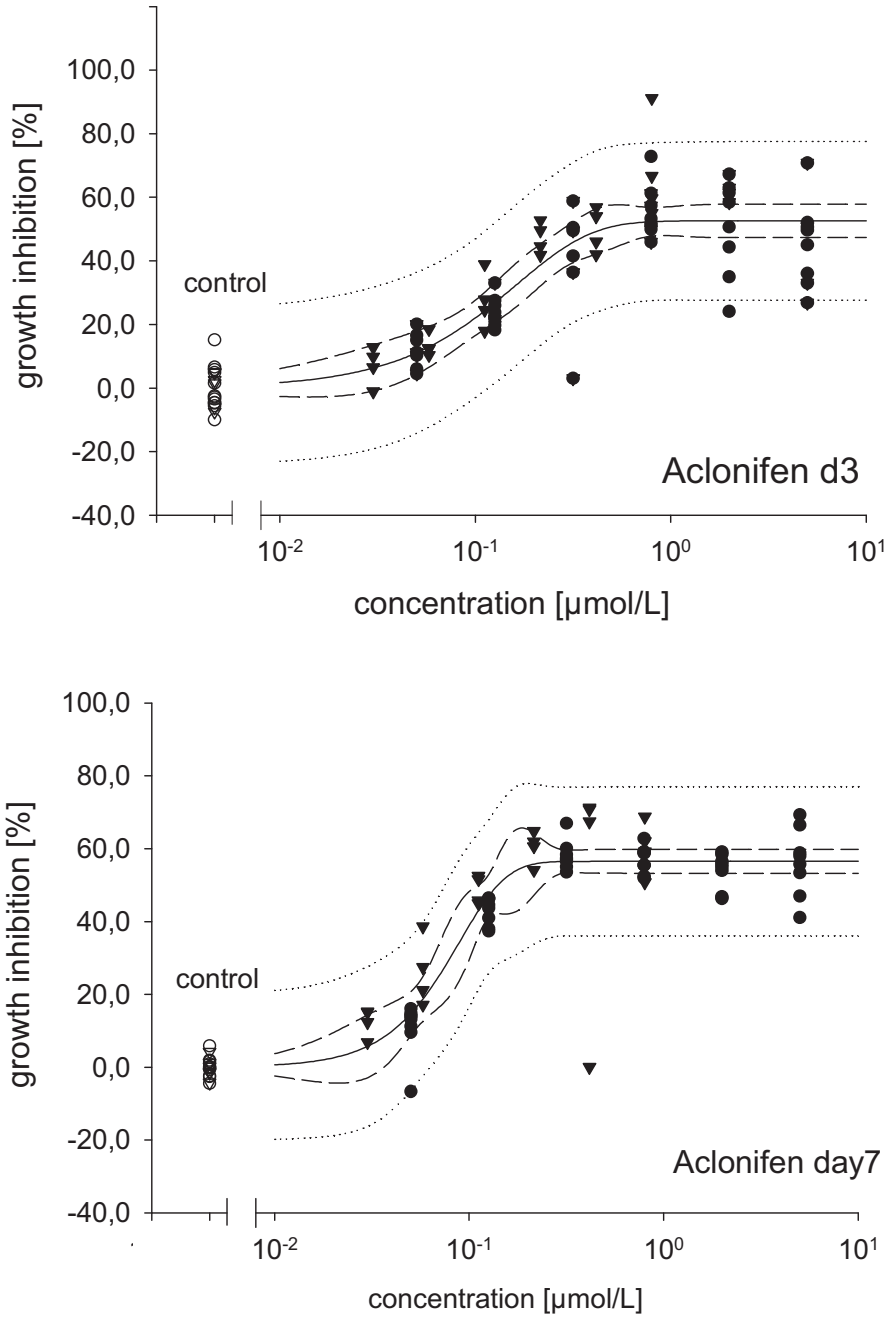


Figure 35A: Dose response curve of Aclonifen at day three and day seven.
Filled symbols denote treated samples, open symbols the untreated controls. Solid line gives the fit to the data, inner dashed lines the approximate 95% confidence belt of the mean, outer dotted lines the 95% confidence belt of the population. Growth was recorded on the basis of the frond area. The various-shaped symbols indicate the different independent tests.

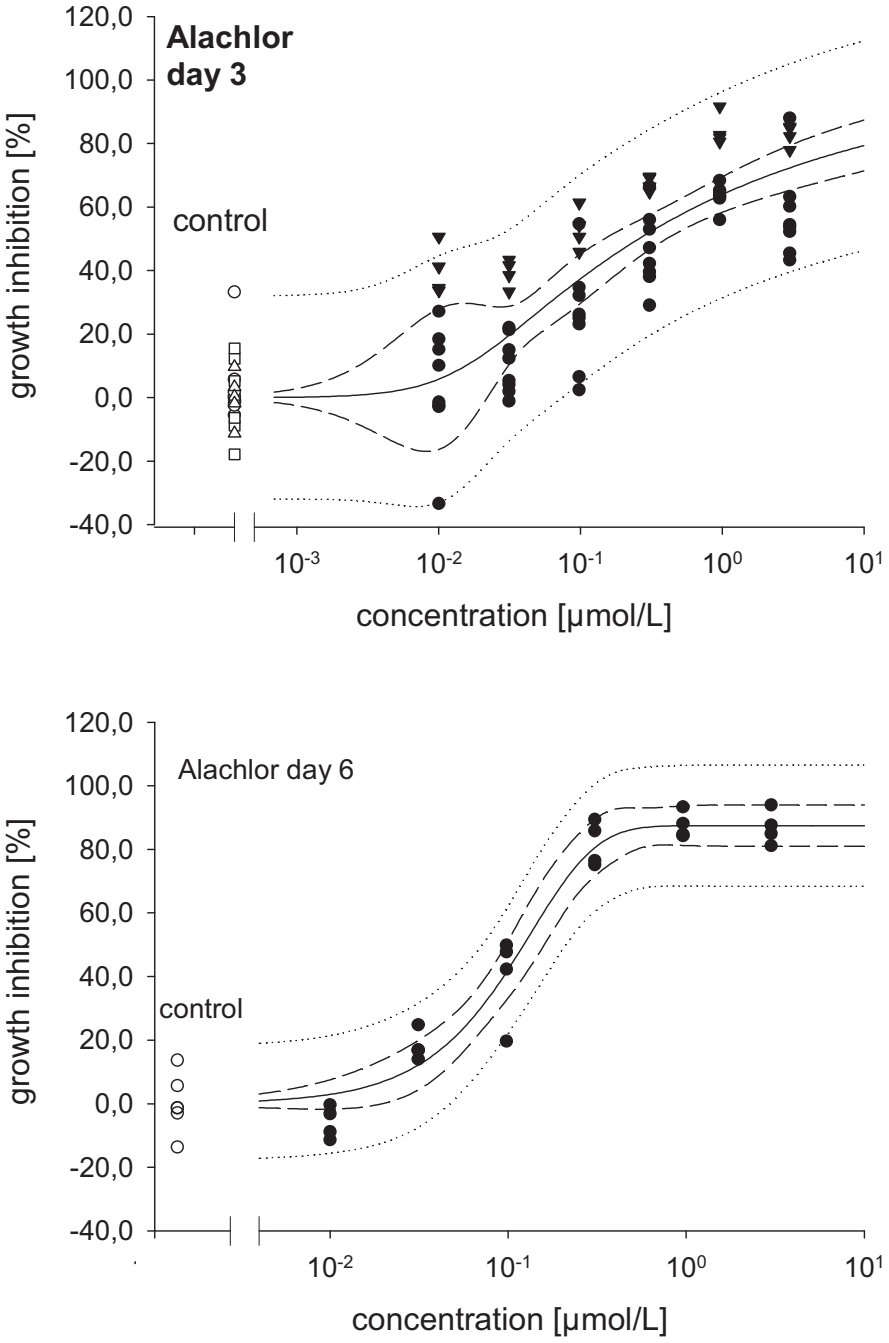


Figure 36A: Dose response curve of Alachlor at day three and day six.
Filled symbols denote treated samples, open symbols the untreated controls. Solid line gives the fit to the data, inner dashed lines the approximate 95% confidence belt of the mean, outer dotted lines the 95% confidence belt of the population. Growth was recorded on the basis of the frond area. The various-shaped symbols indicate the different independent tests.

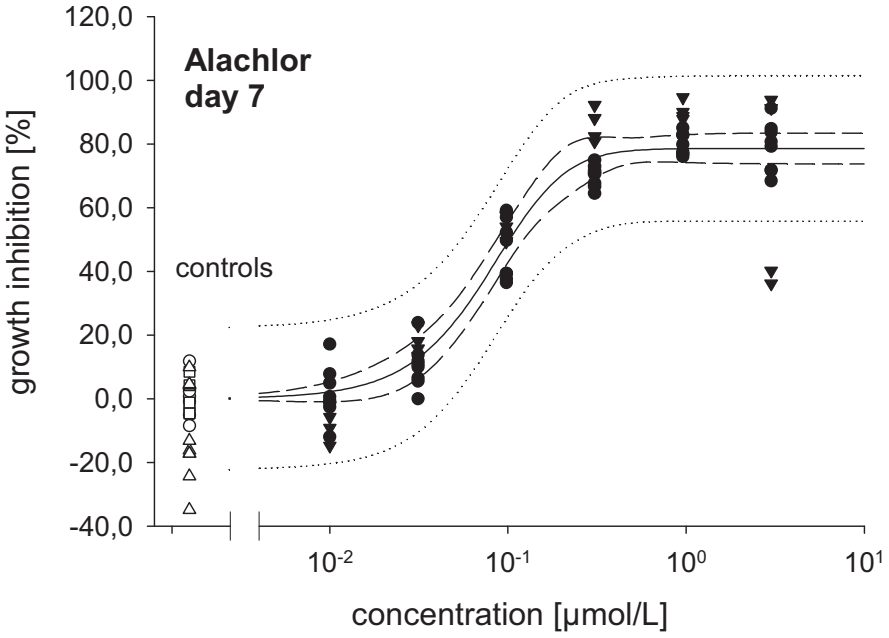


Figure 37A: Dose response curve of Alachlor at day seven.
Filled symbols denote treated samples, open symbols the untreated controls. Solid line gives the fit to the data, inner dashed lines the approximate 95% confidence belt of the mean, outer dotted lines the 95% confidence belt of the population. Growth was recorded on the basis of the frond area. The various-shaped symbols indicate the different independent tests.

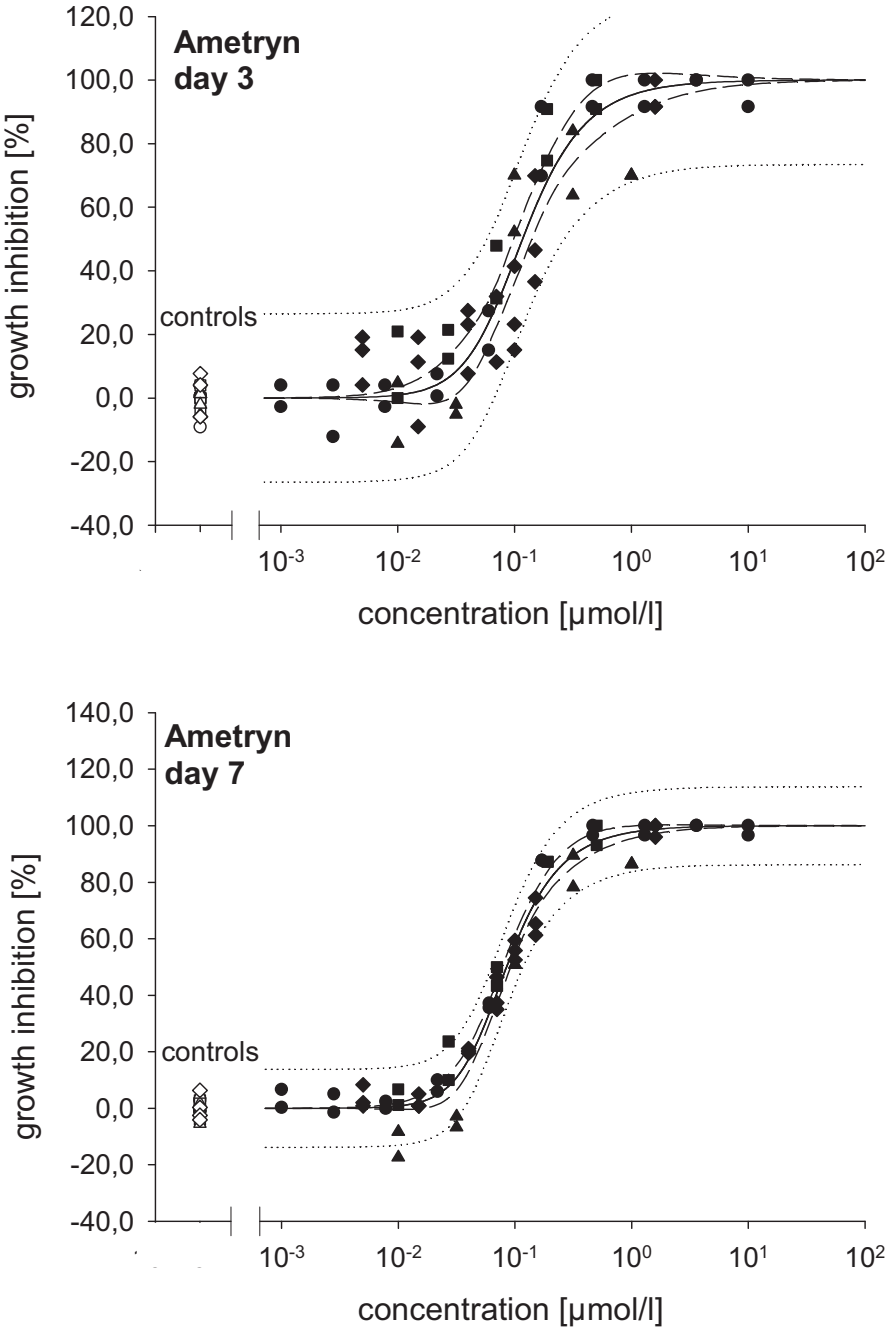


Figure 38A: Dose response curve of Ametryn at day three and day seven.
Filled symbols denote treated samples, open symbols the untreated controls. Solid line gives the fit to the data, inner dashed lines the approximate 95% confidence belt of the mean, outer dotted lines the 95% confidence belt of the population. Growth was recorded on the basis of the frond-number. The various-shaped symbols indicate the different independent tests.

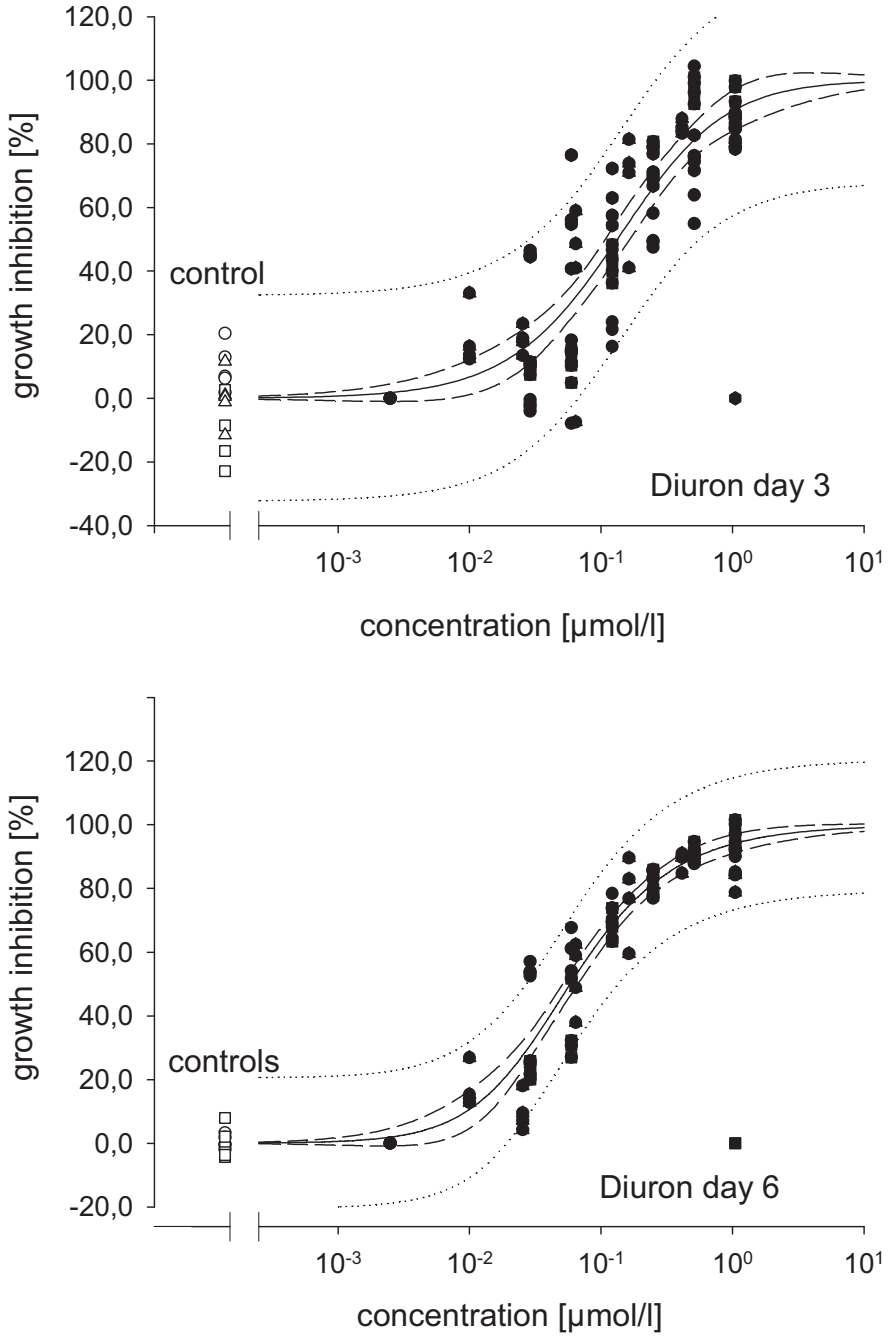


Figure 39A: Dose response curve of Diuron at day three and day six.
Filled symbols denote treated samples, open symbols the untreated controls. Solid line gives the fit to the data, inner dashed lines the approximate 95% confidence belt of the mean, outer dotted lines the 95% confidence belt of the population. Growth was recorded on the basis of the frond area. The various-shaped symbols indicate the different independent tests.

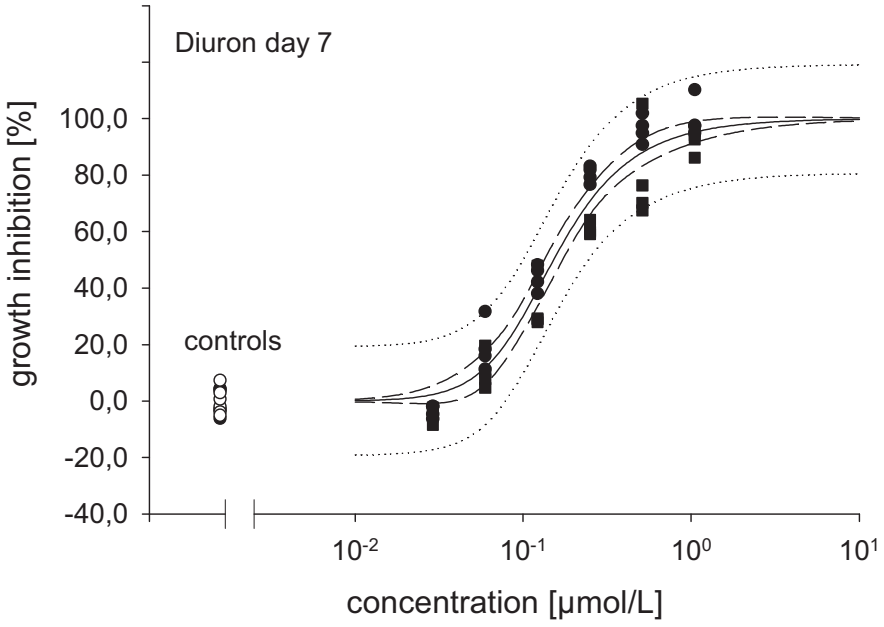


Figure 40A: Dose response curve of Diuron at day seven.
Filled symbols denote treated samples, open symbols the untreated controls. Solid line gives the fit to the data, inner dashed lines the approximate 95% confidence belt of the mean, outer dotted lines the 95% confidence belt of the population. Growth was recorded on the basis of the frond area. The various-shaped symbols indicate the different independent tests.

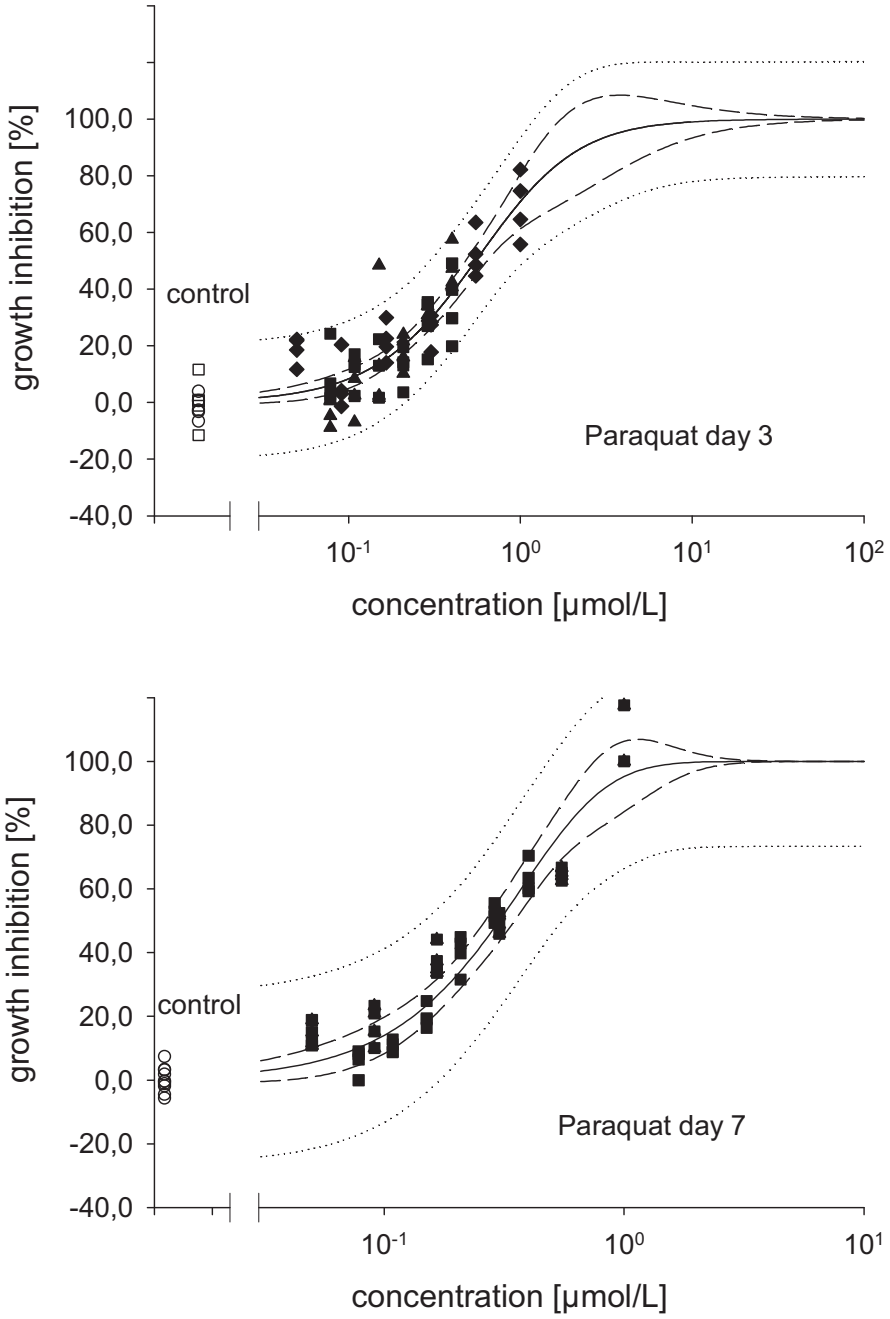


Figure 41A: Dose response curve of Paraquat day three and day seven.
Filled symbols denote treated samples, open symbols the untreated controls. Solid line gives the fit to the data, inner dashed lines the approximate 95% confidence belt of the mean, outer dotted lines the 95% confidence belt of the population. Growth was recorded on the basis of the frond area. The various-shaped symbols indicate the different independent tests.

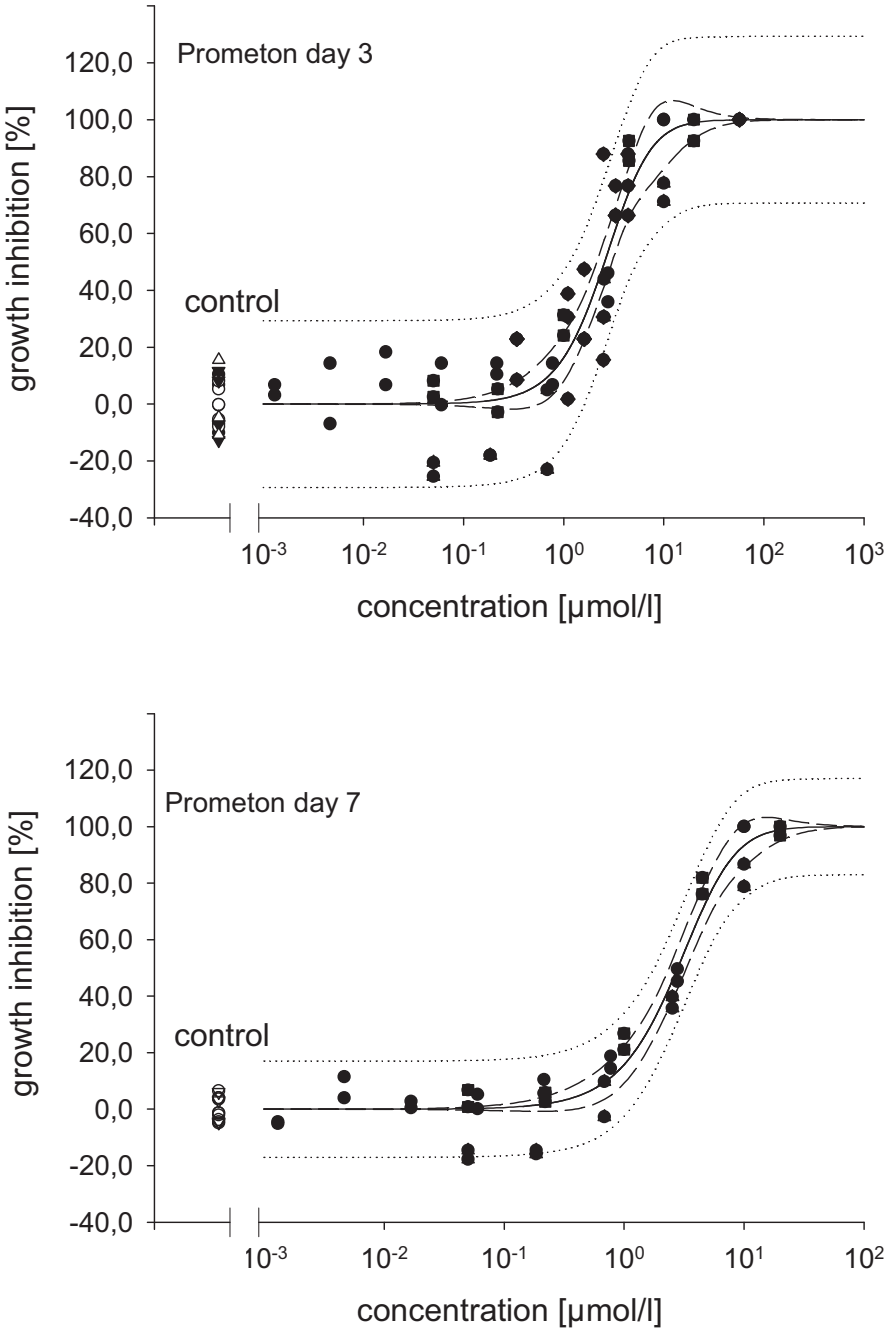


Figure 42A: Dose response curve of Prometon at day three and day seven.
Filled symbols denote treated samples, open symbols the untreated controls. Solid line gives the fit to the data, inner dashed lines the approximate 95% confidence belt of the mean, outer dotted lines the 95% confidence belt of the population. Growth was recorded on the basis of the frond-number. The various-shaped symbols indicate the different independent tests.

Metals

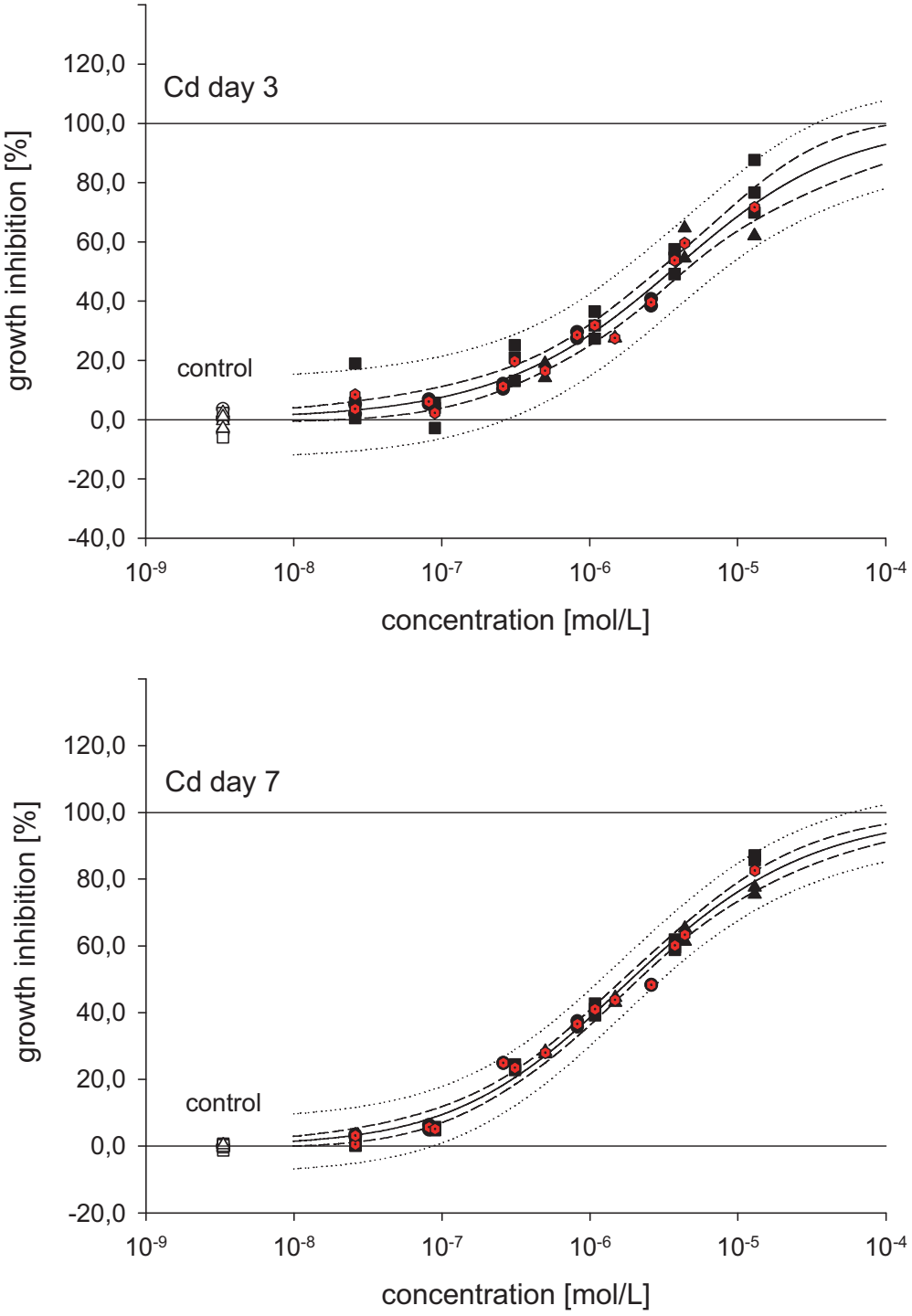


Figure 43A: Dose response curve of cadmium at day three and day seven.
Filled symbols denote treated samples, open symbols the untreated controls. Solid line gives the fit to the data, inner dashed lines the approximate 95% confidence belt of the mean, outer dotted lines the 95% confidence belt of the population. Growth was recorded on the basis of the frond-number. The various-shaped symbols indicate the different independent tests.

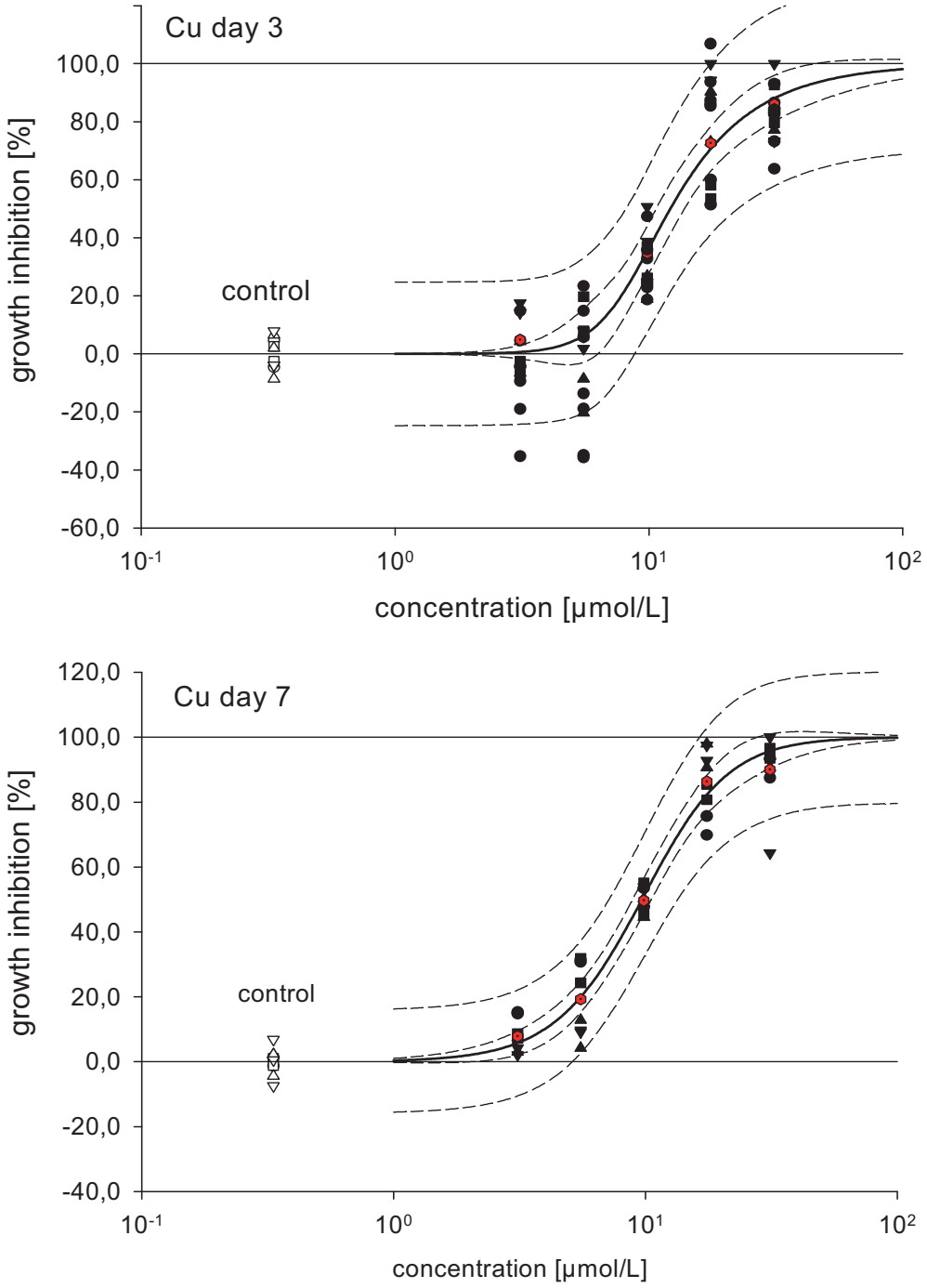


Figure 44A: Dose response curve of copper at day three and day seven.
Filled symbols denote treated samples, open symbols the untreated controls. Solid line gives the fit to the data, inner dashed lines the approximate 95% confidence belt of the mean, outer dotted lines the 95% confidence belt of the population. Growth was recorded on the basis of the frond-number. The various-shaped symbols indicate the different independent tests.

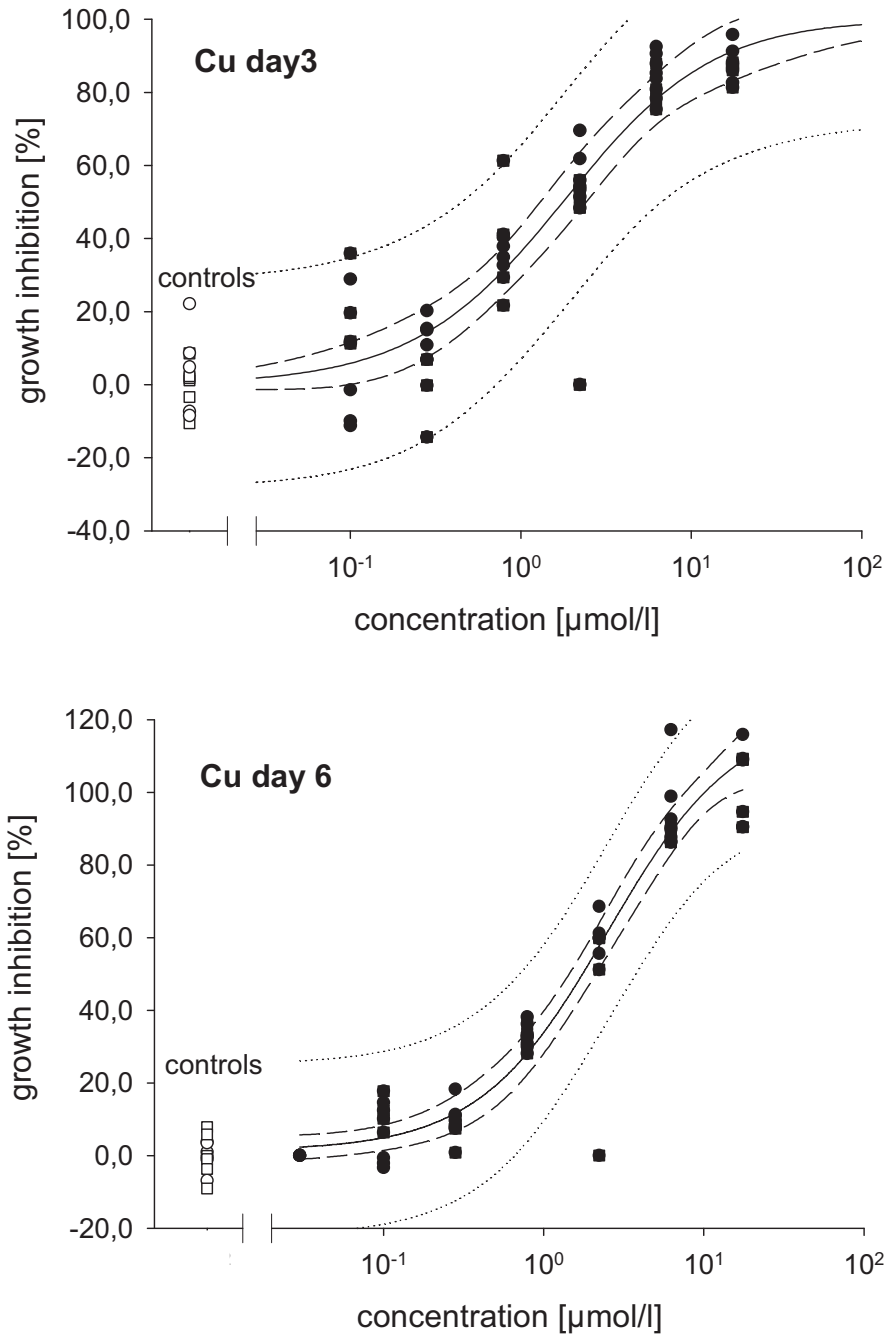


Figure 45A: Dose response curve of copper at day three and day six.
Filled symbols denote treated samples, open symbols the untreated controls. Solid line gives the fit to the data, inner dashed lines the approximate 95% confidence belt of the mean, outer dotted lines the 95% confidence belt of the population. Growth was recorded on the basis of the frond area. The various-shaped symbols indicate the different independent tests.

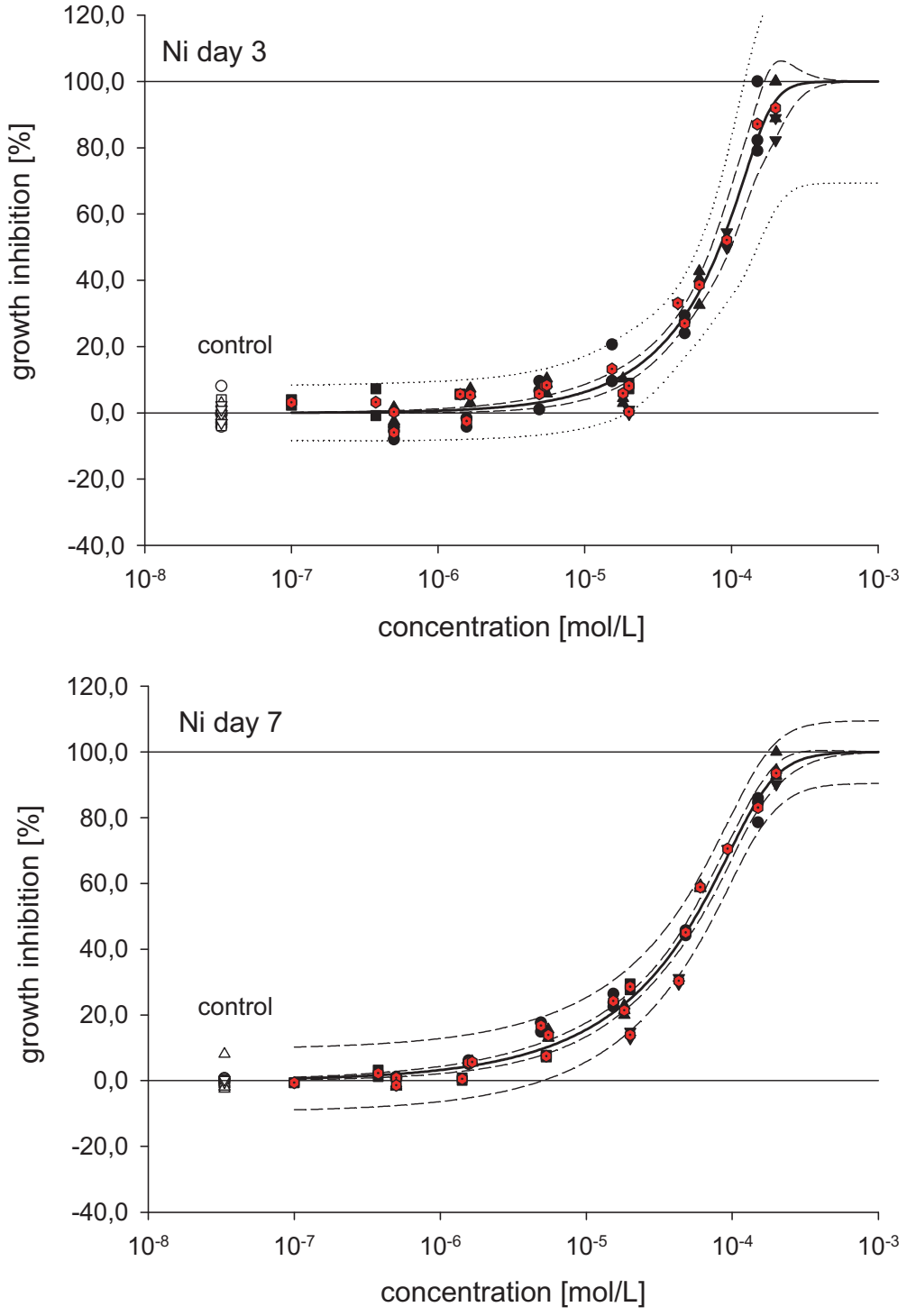


Figure 46A: Dose response curve of nickel at day three and day seven.
Filled symbols denote treated samples, open symbols the untreated controls. Solid line gives the fit to the data, inner dashed lines the approximate 95% confidence belt of the mean, outer dotted lines the 95% confidence belt of the population. Growth was recorded on the basis of the frond-number. The various-shaped symbols indicate the different independent tests.

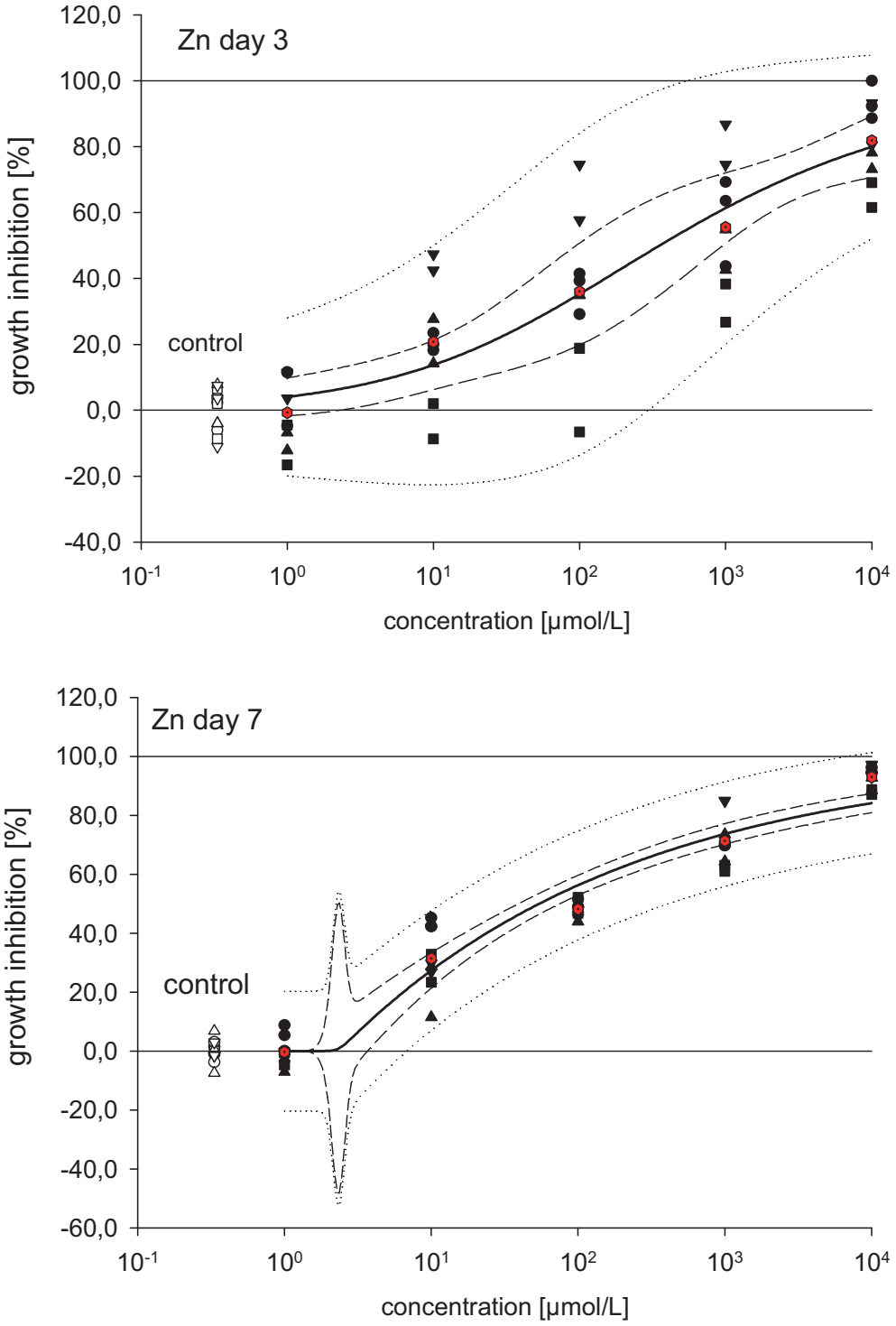
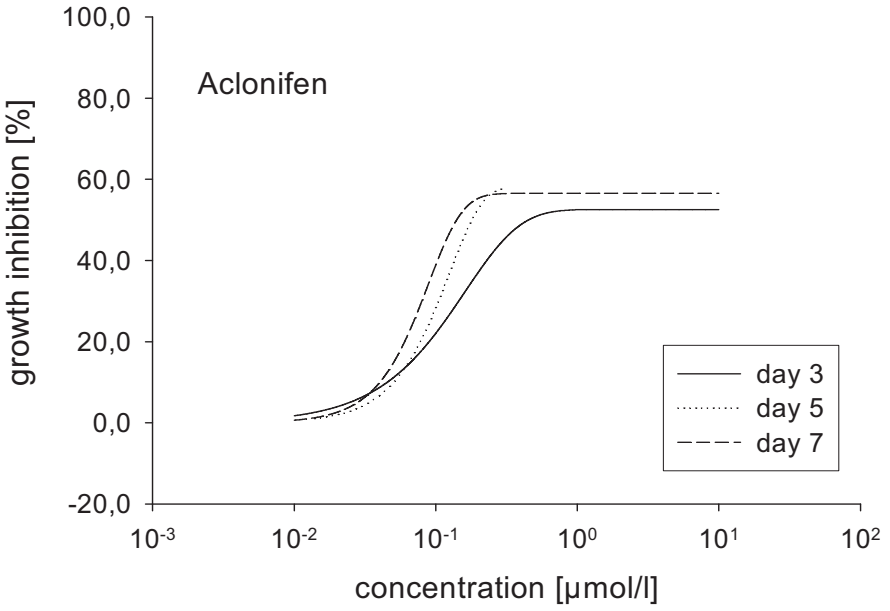


Figure 47A: Dose response curve of zinc at day three and day seven.
Filled symbols denote treated samples, open symbols the untreated controls. Solid line gives the fit to the data, inner dashed lines the approximate 95% confidence belt of the mean, outer dotted lines the 95% confidence belt of the population. Growth was recorded on the basis of the frond-number. The various-shaped symbols indicate the different independent tests.

Concentration response curves over time

Herbicides



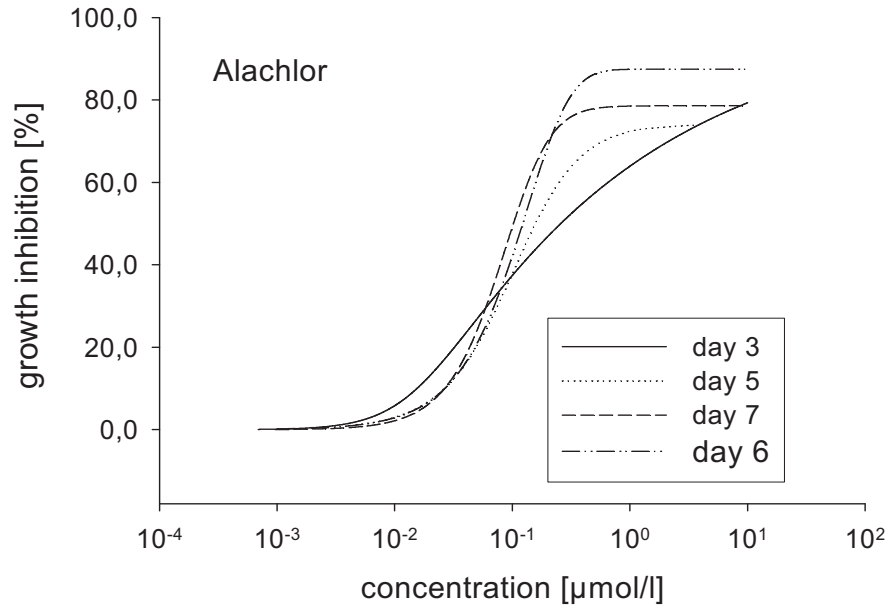


Figure 48A: Development of the concentration response relationship of Aclonifen and
Figure 49A: Development of the concentration response relationship of Alachlor over time.
To illustrate the change of the dose-response relationship over time, all dose-response curves of from day three to day seven are plotted in one graph for each substance respectively.

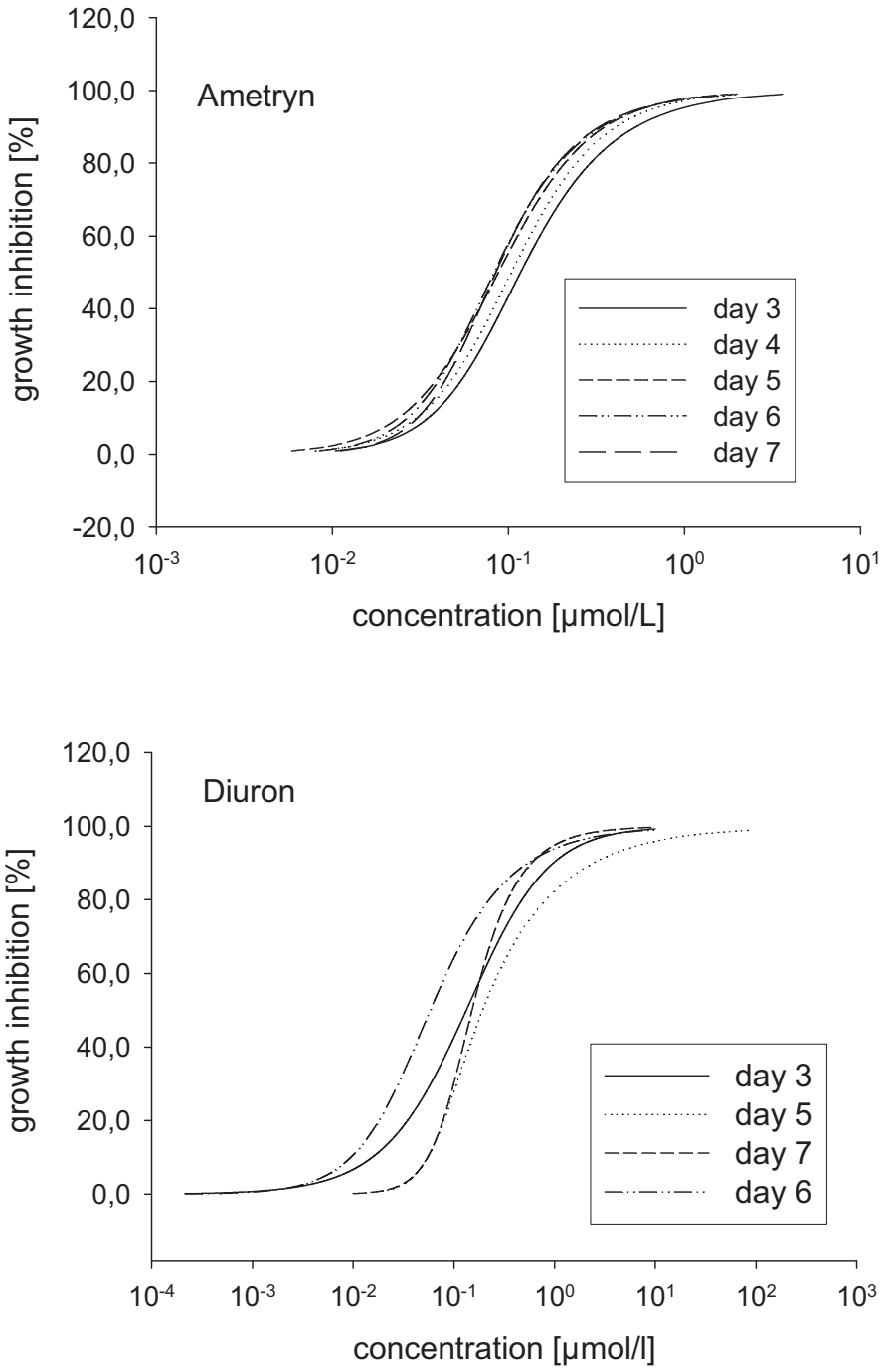


Figure 50A: Development of the concentration response relationship of Ametryn over time.
Figure 51A: Development of the concentration response relationship of Diuron over time.
To illustrate the change of the dose-response relationship over time, all dose-response curves of from day three to day seven are plotted in one graph for each substance respectively.

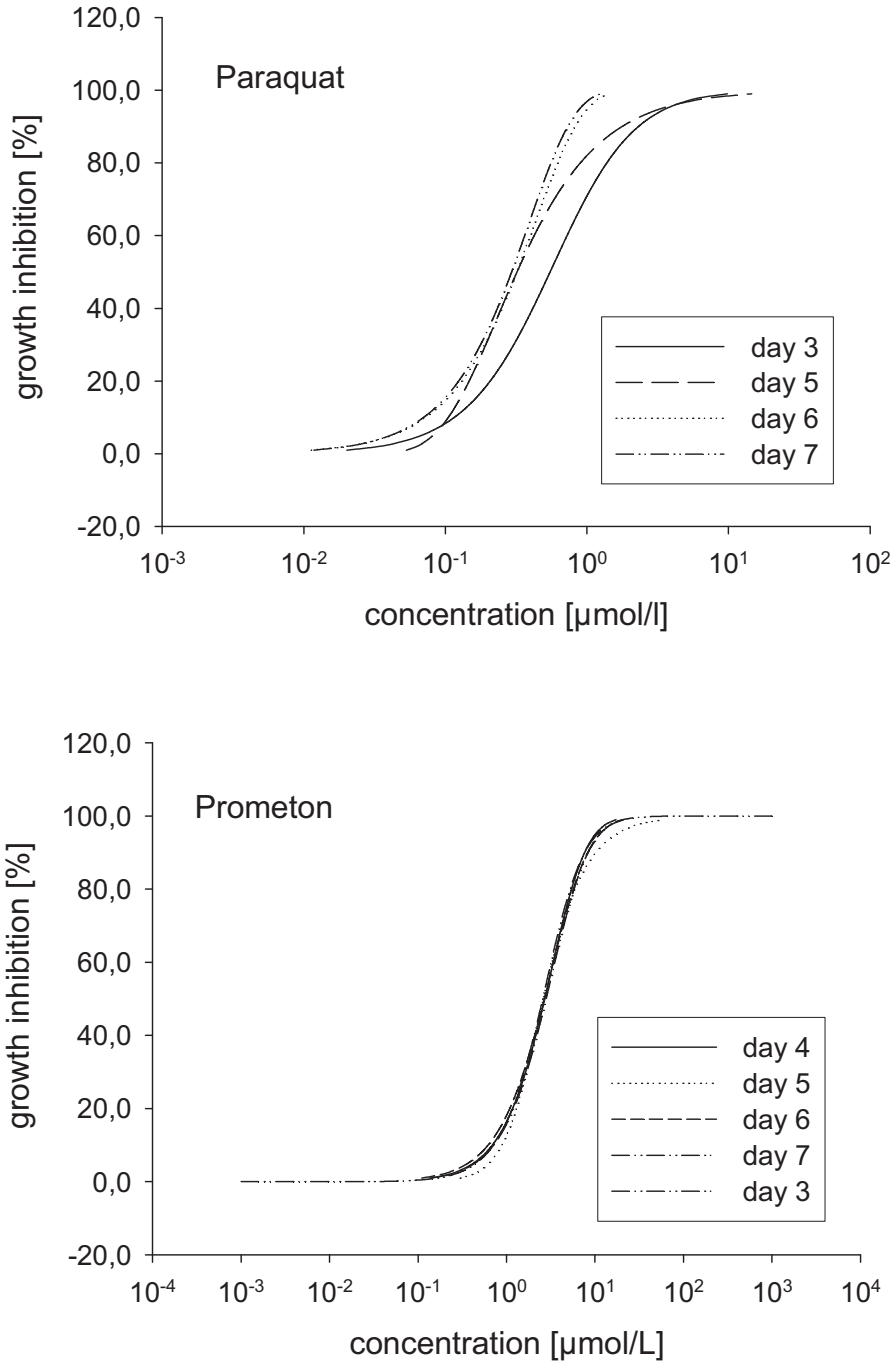
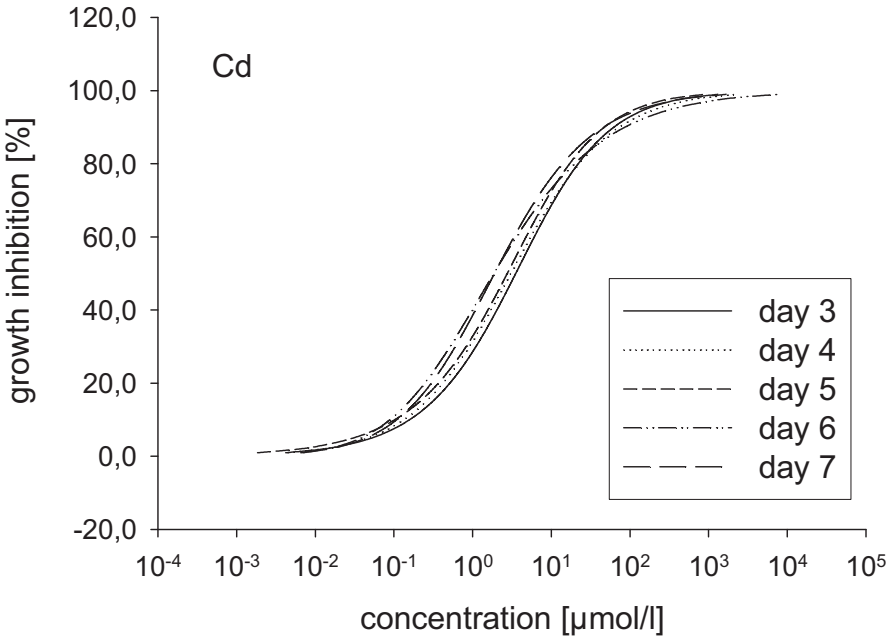


Figure 52A: Development of the concentration response relationship of Paraquat over time.
Figure 53A: Development of the concentration response relationship of Prometon over time.
To illustrate the change of the dose-response relationship over time, all dose-response curves of from day three to day seven are plotted in one graph for each substance respectively.

Metals



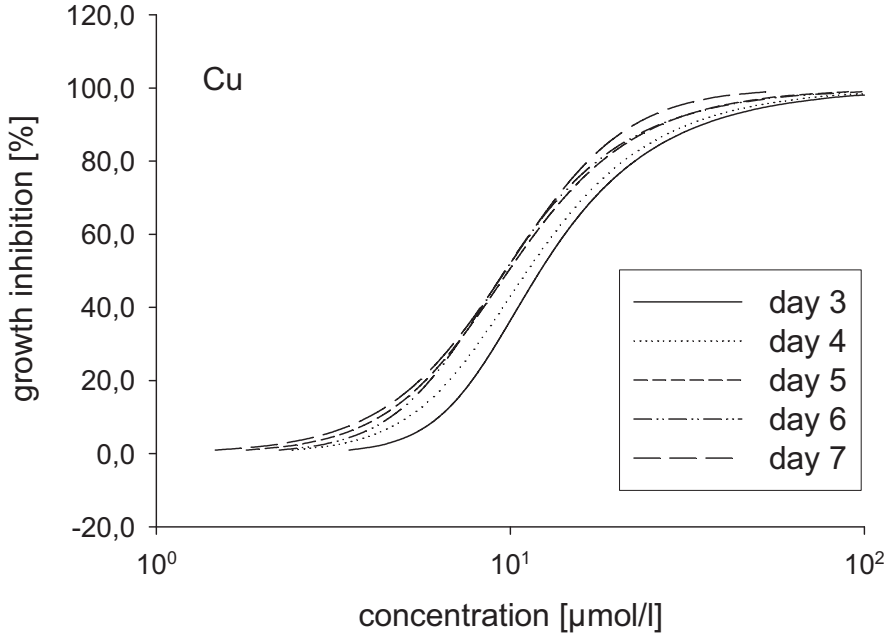
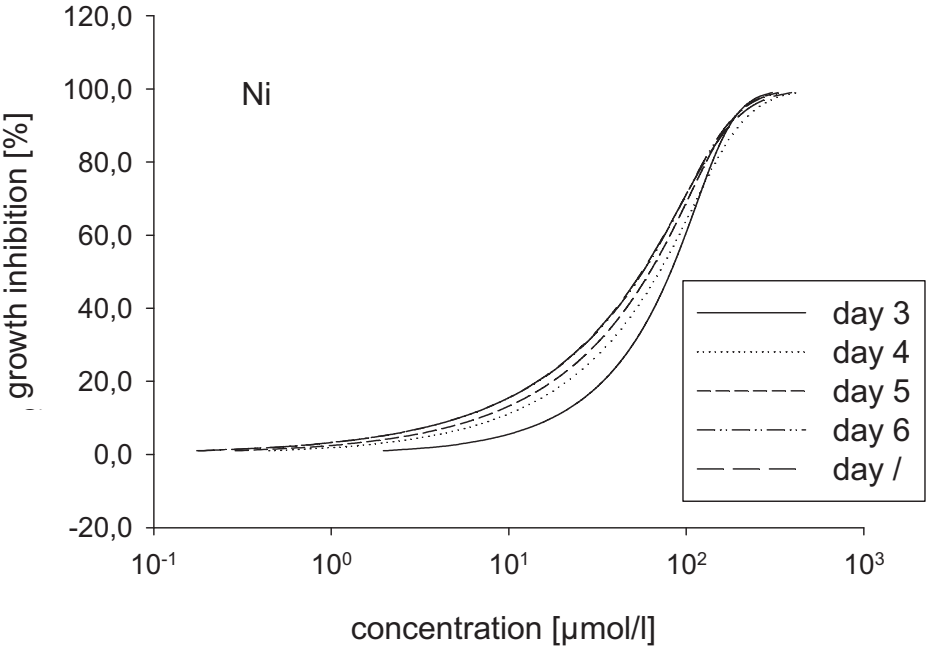


Figure 54A: Development of the concentration response relationship of cadmium over time.
Figure 55A: Development of the concentration response relationship of copper over time.
To illustrate the change of the dose-response relationship over time, all dose-response curves of from day three to day seven are plotted in one graph for each substance respectively.



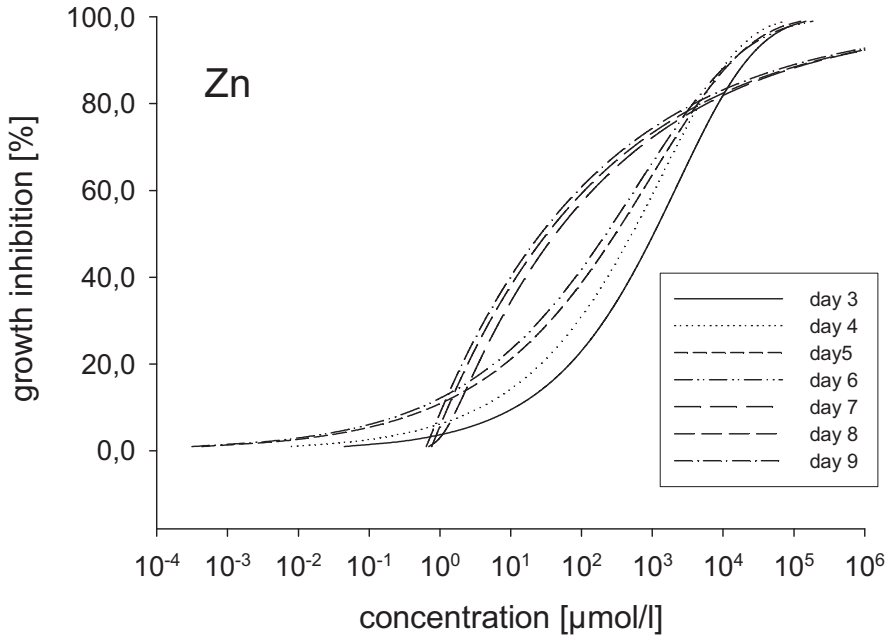


Figure 56A: Development of the concentration response relationship of nickel over time.
Figure 57A: Development of the concentration response relationship of zinc over time.
To illustrate the change of the dose-response relationship over time, all dose-response curves of from day three to day seven are plotted in one graph for each substance respectively.

Development of the toxicity over time, fitted with the equations of Haber, Bliss and Ostwald/Dernoscheck

Herbicides

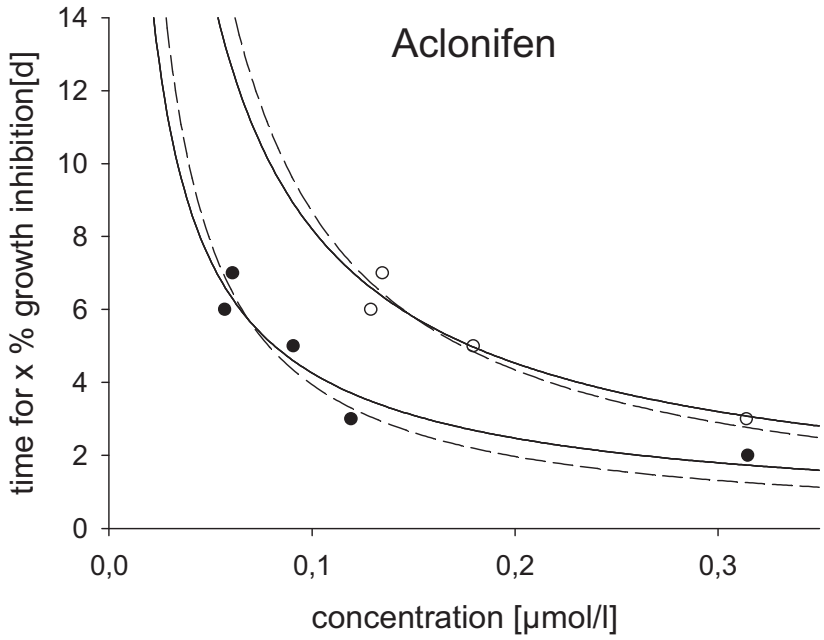
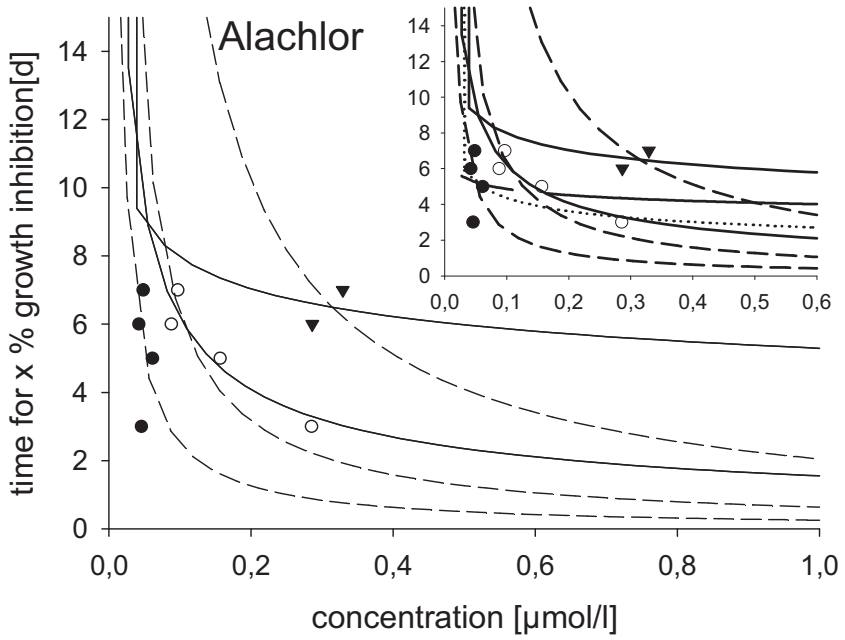


Figure 58A: Toxicity of Aclonifen over time fitted with the equations of Haber, Bliss and Ostwald/Dernoscheck..
. The black circles represent the EC_{25} values, white circles the EC_{50} values and the black triangles the EC_{75} values derived from the concentration-response curves of single substance tests over time from three up to seven days of exposure. The EC values were fitted with either the Haber equation ($c \cdot t = k$) shown here as a dashed line, the Bliss equation ($c \cdot t^{\gamma} = k$) shown as a dotted line or the Ostwald/Dernoscheck equation ($c^{\gamma} \cdot t = k$) shown as a solid line. Generally the curve-progressions of the Bliss and the Ostwald/Dernoscheck fits are identical and thus the curves overlapp.



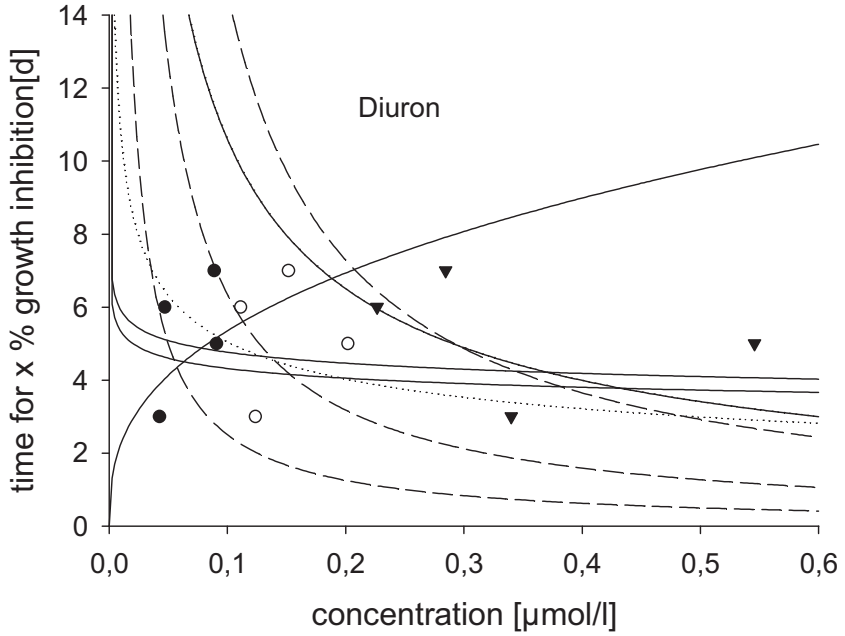


Figure 59A: Toxicity of Diuron over time fitted with the equations of Haber, Bliss and Ostwald/Dernoscheck..

Figure 60A: Toxicity of Alachlor over time fitted with the equations of Haber, Bliss and Ostwald/Dernoscheck..

The black circles represent the EC_{25} values, white circles the EC_{50} values and the black triangles the EC_{75} values derived from the concentration-response curves of single substance tests over time from three up to seven days of exposure. The EC values were fitted with either the Haber equation ($c \cdot t = k$) shown here as a dashed line, the Bliss equation ($c \cdot t^{\gamma} = k$) shown as a dotted line or the Ostwald/Dernoscheck equation ($c^{\gamma} \cdot t = k$) shown as a solid line. Generally the curve-progressions of the Bliss and the Ostwald/Dernoscheck fits are identical and thus the curves overlapp.

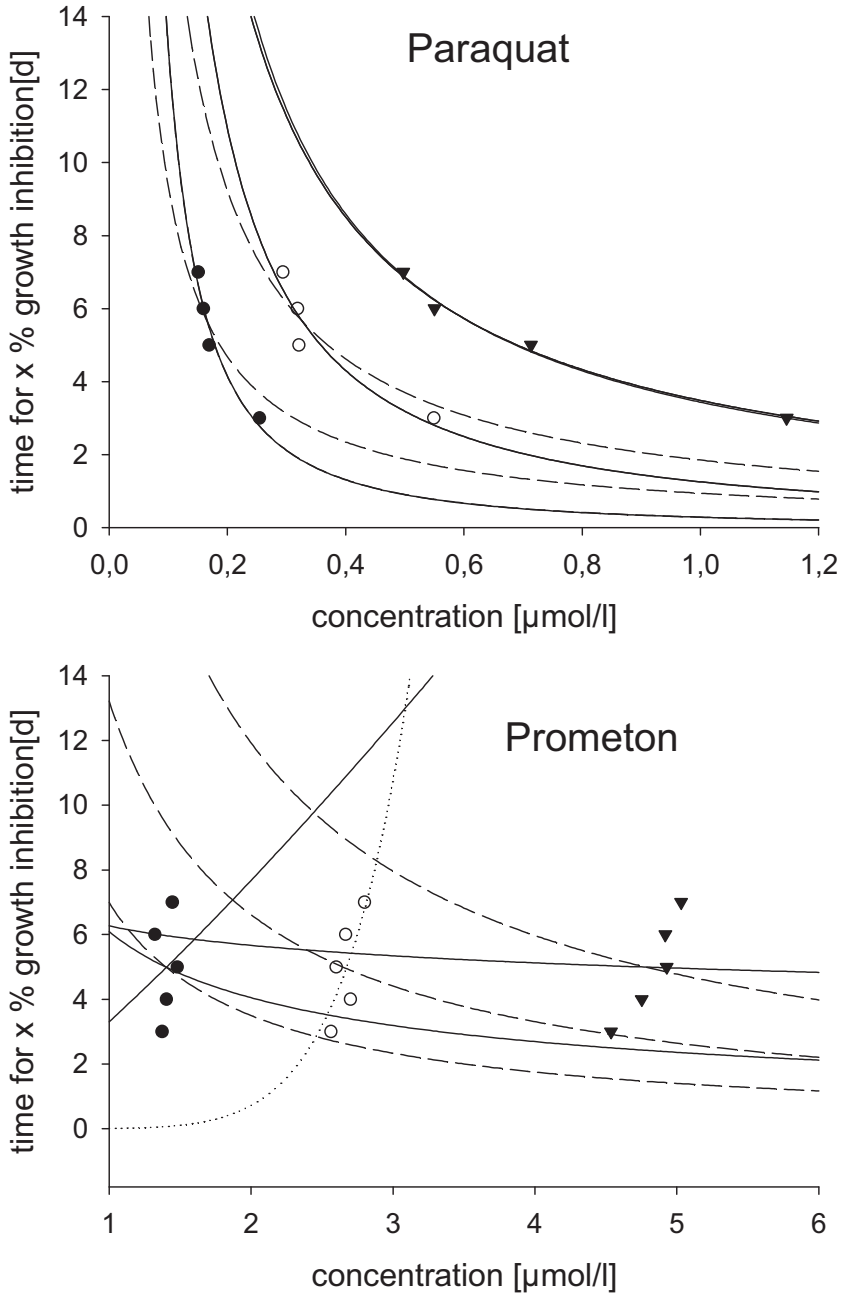


Figure 61A: Toxicity of Paraquat over time fitted with the equations of Haber, Bliss and Ostwald/Dernoscheck.
Figure 62A: Toxicity of Prometon over time fitted with the equations of Haber, Bliss and Ostwald/Dernoscheck.
The black circles represent the EC_{25} values, white circles the EC_{50} values and the black triangles the EC_{75} values derived from the concentration-response curves of single substance tests over time from three up to seven days of exposure. The EC values were fitted with either the Haber equation ($c \cdot t = k$) shown here as a dashed line, the Bliss equation ($c \cdot t^{\gamma} = k$) shown as a dotted line or the Ostwald/Dernoscheck equation ($c^{\gamma} \cdot t = k$) shown as a solid line. Generally the curve-progressions of the Bliss and the Ostwald/Dernoscheck fits are identical and thus the curves overlapp.

Metals

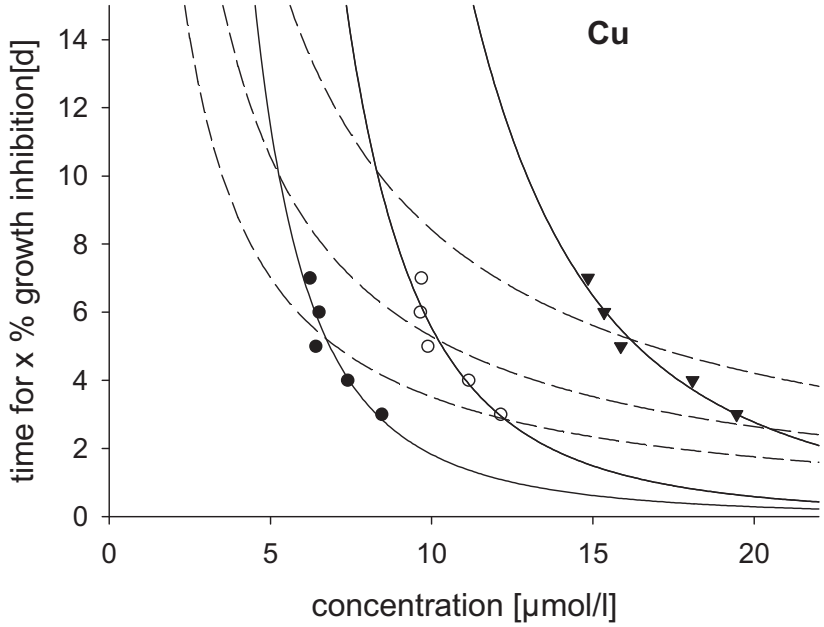


Figure 63A: Toxicity of copper over time fitted with the equations of Haber, Bliss and Ostwald/Dernoscheck.
The black circles represent the EC_{25} values, white circles the EC_{50} values and the black triangles the EC_{75} values derived from the concentration-response curves of single substance tests over time from three up to seven days of exposure. The EC values were fitted with either the Haber equation ($c \cdot t = k$) shown here as a dashed line, the Bliss equation ($c \cdot t^{\gamma} = k$) shown as a dotted line or the Ostwald/Dernoscheck equation ($c^{\gamma} \cdot t = k$) shown as a solid line. Generally the curve-progressions of the Bliss and the Ostwald/Dernoscheck fits are identical and thus the curves overlapp.

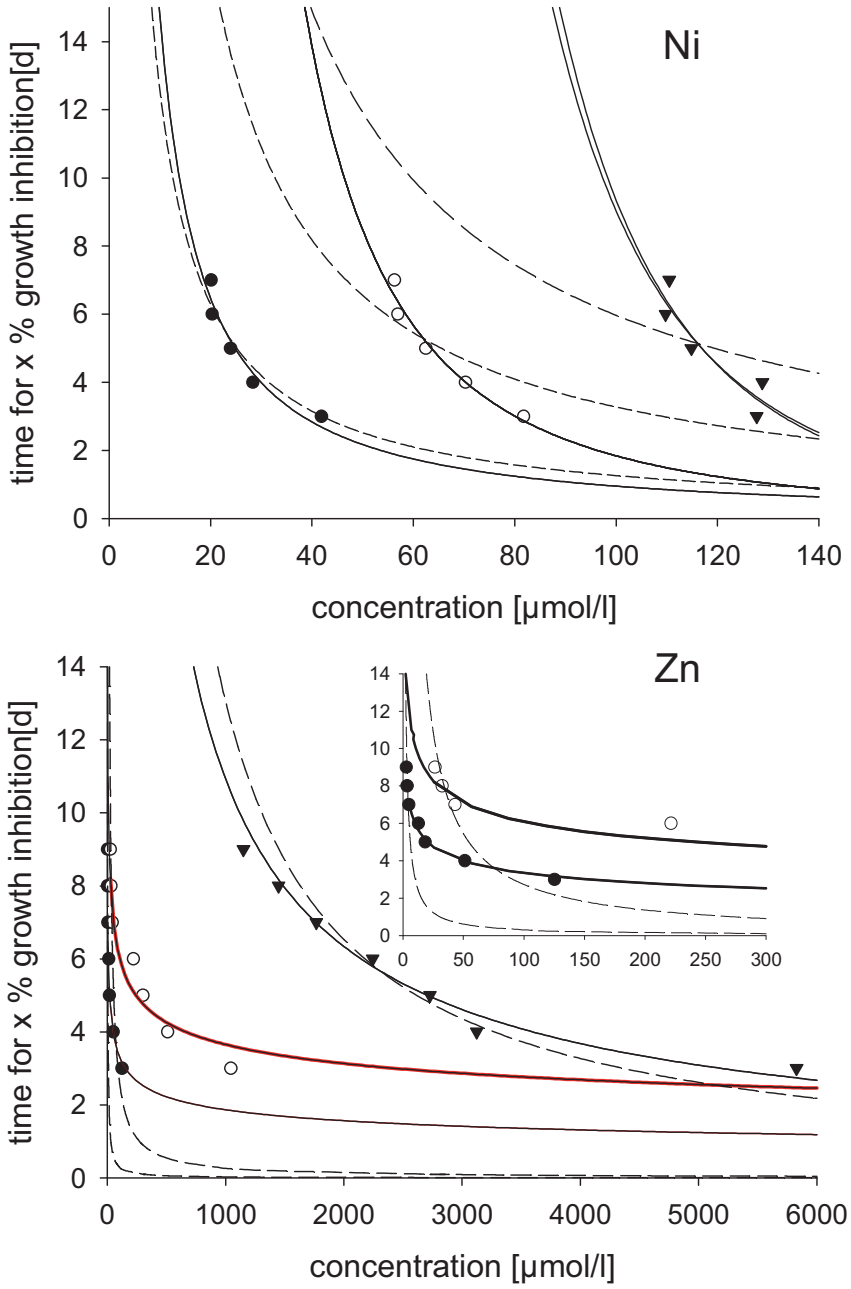


Figure 64A: Toxicity of nickel over time fitted with the equations of Haber, Bliss and Ostwald/Dernoscheck..

Figure 65A: Toxicity of zinc over time fitted with the equations of Haber, Bliss and Ostwald/Dernoscheck.. The black circles represent the EC_{25} values, white circles the EC_{50} values and the black triangles the EC_{75} values derived from the concentration-response curves of single substance tests over time from three up to seven days of exposure. The EC values were fitted with either the Haber equation ($c \cdot t = k$) shown here as a dashed line, the Bliss equation ($c \cdot t^{\gamma} = k$) shown as a dotted line or the Ostwald/Dernoscheck equation ($c^{\gamma} \cdot t = k$) shown as a solid line. Generally the curve-progressions of the Bliss and the Ostwald/Dernoscheck fits are identical and thus the curves overlapp.

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List of scientific contributions

Articles:

Drost W, Backhaus T, Vassilakaki M, Grimme LH (2003): Mixture of s-triazines toxicity to *Lemna* minor under conditions of simultaneous and sequential exposure. Fresenius Environmental Bulletin 12(6): 601-607

Drost W, Matzke M, Backhaus T (2007): Heavy metal toxicity to *Lemna* minor: studies on the time-dependence of growth inhibition and the recovery after exposure. Chemosphere 67(1): 36-43

Junghans M, Schaefer M, Drost W, Hassold E, Stock F, Dünne M, Juffernholz T, Meyer M, Ranke J (2008): Reconsidering Environmental Effects Assessment of Chemicals: proposal for a dynamic testing strategy. Basic and Applied Ecology 9(4): 356-364

Ahlf W, Drost W, Heise S (2009): Incorporation of metal bioavailability into regulatory frameworks—metal exposure in water and sediment. Journal of Soils and Sediments 9(5): 411-419

Ehrlich G, Jöhncke U, Drost W, Schulte C (2011): Problems Faced when Evaluating the Bioaccumulation Potential of Substances under REACH. Integrated Environmental Assessment and Management doi: 10.1002/ieam

Conference contributions

Oral presentations:

Drost W, Junghans M, Backhaus T, Dünne M, Frische T, Hassold E, Meyer W, Mölter K, Ranke J, Schaefer M, Stock F (2003): Biotests zur ökotoxikologischen Gefährdungsabschätzung von Chemikalien: Wissenschaftlicher Anspruch versus Pragmatismus.Workshop „Ecotoxicology and Ecosystems: Relevance, Restrictions, Research and Needs“of the Arbeitskreis Ökosystemforschung der GfÖ, Bredbeck, Germany

T Juffernholz , W Drost, E Hassold, N Aust (2010) How to assess and communicate the risks of chemical mixtures – different points of view. 20th Annual Meeting of SETAC Europe (Society of Environmental Toxicology and Chemistry) Sevilla, Spain

Posters:

Drost W, Backhaus T, Vassilikaki M, Grimme LH (2002): Mixture toxicity of s-triazines to *Lemna* minor under conditions of simultaneous and sequential exposure. Annual meeting 2002 of the GDCh (Gesellschaft deutscher Chemiker), SETAC-GLB (Society of Environmental Toxicology and Chemistry, German Language Branch) and the Verband für Geoökologie in Deutschland e.V, Braunschweig, Germany

"Young scientist award" for the best poster in the session „Aquatische und Terrestrische Ökosysteme – Ökotoxikologische Testsysteme"

Drost W, Backhaus T, Grimme LH (2003): Mixture toxicity of heavy metals to *Lemna* minor under conditions of simultaneous and sequential exposure. 13th Annual Meeting of SETAC Europe (Society of Environmental Toxicology and Chemistry) Hamburg, Germany

Drost W, Backhaus T, Grimme LH (2003): Mischungstoxizität von Kupfer und Zink bei simultaner und sequenzieller Exposition auf *Lemna* minor. 8th annual meeting of SETAC-GLB (Society of Environmental Toxicology and Chemistry, German Language Branch), Heidelberg, Germany

Drost W, Backhaus T, Matzke M, Grimme LH (2004): The time dependence of heavy metal toxicity. 14th Annual Meeting of SETAC Europe (Society of Environmental Toxicology and Chemistry) Prag, Tschechische Republik

Drost W, Backhaus T, Matzke M, Grimme LH (2004): The time dependence of heavy metal toxicity in *Lemna* minor. Workshop, Internal exposure-linking bioavailability to effects, organised by the EAWAG (Swiss Federal Institute for Environmental Science and Technology), Universität Utrecht and ETH Zürich in Monte Verita, Switzerland

Drost W, Backhaus T, Matzke M (2005): Pulsed and sequential exposure of heavy metals and herbicides: studies of cumulative toxicity on *Lemna* minor. 15th Annual Meeting of SETAC Europe (Society of Environmental Toxicology and Chemistry) Lille, France

Drost W, Backhaus T (2009) Sequential exposure to binary mixtures of copper, Diuron and Alachlor - Toxicity studies with *Lemna* minor. 19th Annual Meeting of SETAC Europe (Society of Environmental Toxicology and Chemistry) Göteborg, Sweden

Curriculum vitae

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